DIAGNOSTIC PROCEDURES FOR VIRUS AND RICKETTSIAL DISEASES



Second Edition

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PREFACE

TO ATTAIN a completely current status for a book dealing with technical procedures in a field of rapid growth is difficult. The last few years have seen the remarkable extension of tissue culture methods in isolation and identification of viruses of human disease. Consequently, diagnostic procedures for poliomyelitis have become beautiful laboratory technics instead of limited, burdensome undertakings in experimental animals. The large groups of adenoviruses and echo viruses have been disclosed by these means Measles, chicken pox, herpes zoster—viruses which for years were on the threshold of demonstration have been identified Interesting immunologic interrelationships between viruses have been established during this period so that the view is increasingly one of families of viruses and rickettsiae rather than of multiple unrelated entities Established serologic technics have been improved, refined, and adapted to more precise measurements

The eight-year period since the publication of the first edition of *Diagnostic Procedures for Virus and Rickettsial Diseases* has been one of extensive development in the facilities and personnel engaged in application of diagnostic methods to clinical and epidemiologic investigations of virus and rickettsial diseases The present edition was originally considered nearly five years ago, but varied factors resulted in its postponement Then the schedule was set for publication in 1954 In the meantime both the editors and a number of the original authors became committed to new duties which delayed or prevented their participation The delays have, however, permitted the inclusion of new material in the book

This second edition retains as its objective the presentation of technical diagnostic procedures It is again primarily the work of authors who are authorities on the respective subjects but who are in addition leading investigators and active designers of the diagnostic procedures Despite their extended activities, they have borne with patience the delays and have co-operated remarkably in making extensive revisions to meet the changing situation Little effort has been made by the editors to impose conformity of wording or to suggest uniformity of technic The book is not one of research methods, and in most instances

the procedures emphasized are those considered most useful and practicable in diagnosis of the disease entity.

In addition to the chapters that comprise the first edition, there are four new ones. The introductory chapter by Edwin H Lennette presents the General Principles Underlying Laboratory Diagnosis. Another general section is Tissue Culture Methods for the Cultivation of Poliomyelitis and Other Viruses prepared by Joseph L Melnick. Maurice R. Hilleman has contributed the chapter on Miscellaneous Virus Diseases, recognizing that the diagnostic procedures for many of these twenty diseases may be moving into positions of greater prominence as information develops Coxsackie Viruses was written by Gilbert Dalldorf and Grace M Sickles. The adenoviruses are primarily considered in the chapter on Atypical Pneumonia. There is a brief introduction to the echo viruses Some new names appear in this edition as authors or co-authors. Karl Habel collaborated with John Enders in the chapter on Mumps. Dr. Hilleman joined with A E Feller on Atypical Pneumonia. The chapter on Influenza was revised by Keith Jensen, and C. H Kempe rewrote the one on Variola and Vaccinia Joseph E Smadel has redone the entire section on Rickettsial Diseases and has contributed the large diagnostic charts. In addition, he has served as associate editor of the volume All of them and the continuing authors of the first edition constitute the Committee on Diagnostic Procedures for Virus and Rickettsial Diseases, which presents this revised and enlarged edition

Mrs Genevieve S Reilly has cheerfully and effectively carried the brunt of the work as editorial assistant Miss Helen H. Wild has again thoroughly verified the references The Ann Arbor Press, Mr. Beatty, manager, has been most helpful and efficient concerning format and typographic details

THOMAS FRANCIS, JR, M D, *Chairman*
Committee on Diagnostic Procedures
for Virus and Rickettsial Diseases,
American Public Health Association

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LABOR
VIRUS AND

EDWIN H LENNETTE, M.D , PH.D

Chief of the Viral and Rickettsial Disease Laboratory,
Division of Laboratories,
California State Department of Public Health,
Berkeley, California

a Reed-Muench method
b. Kärber method
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a Reed-Muench method
b Kärber method

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I. INTRODUCTION

A. GENERAL CONSIDERATIONS

IN THE ensuing chapters there is presented specific and detailed information concerning the methods and technics utilized in arriving at a laboratory diagnosis of a given disease or group of diseases. The reader, through careful perusal of this book or through actual experience, will recognize that certain technics are simple and inexpensive, whereas others are cumbersome or intricate or costly. The inclusion of methods falling into the latter categories is necessitated by the dual purpose of this book, namely, to provide a working guide for those more or less unfamiliar with virology and rickettsiology but who wish to undertake diagnostic work, as well as to provide a source of quickly accessible detailed information for those already conversant with these fields. This chapter, however, has been prepared primarily for those unfamiliar with virology, in an attempt to outline the general considerations bearing directly on applied diagnosis, and to tie together, in summary form in one place, the various methods and technics described or referred to in sundry later chapters

The main function of the virus diagnostic laboratory is to provide the means for achieving a diagnosis, and it is obvious that those methods which are simple, rapid, and inexpensive and which permit the expeditious examination of large numbers of specimens are the most suitable. Ideally, however, the diagnostic laboratory should be in a position to assist in the investigation of infectious diseases, dependent upon the extent of its participation in such inquiries, the scope of its methodology will be correspondingly broadened and will perforce have to include some of the less simple technics. Despite this research aspect, however, the problems and the operating philosophy of a diagnostic laboratory differ from those of a laboratory whose primary function is research [1]

The bacteriologist or serologist undertaking viral diagnostic work will

find that the *modus operandi* differs from that to which he is accustomed, even though the same basic technics with which he is familiar are utilized Thus, in viral serologic work a diagnosis is seldom made on the basis of a single specimen of blood, usually, multiple specimens of blood from the same patient are tested simultaneously, which means that the early serum specimens must be stored until all the specimens are available for examination. Also, since the sera must be stored for some days or weeks, all specimens are handled with sterile precautions In addition, the serologic tests employ comparatively small volumes of reagents, including serum, as a battery of tests rather than a single test must frequently be done, it is important to conserve the small amount of material generally received by using the minimal amounts compatible with accuracy

Since specific details of the various technics and procedures are given elsewhere in this book, it is not the intention here to describe each method It is intended, rather, to present a general consideration of some of these methods with respect to their use, the proper collection and handling of material which is to undergo examination, and the equipment required for processing and handling these materials in the laboratory and for their examination

II BASIC APPROACHES TO THE DIAGNOSIS OF INFECTIONS CAUSED BY VIRUSES AND RICKETTSIAE

In attempting a diagnosis by laboratory methods, there are essentially three lines of approach

1 Examination of the infected tissues for pathologic alterations,
2. Isolation and identification of the inciting agent, and
3. Demonstration of the appearance, or of a rise in titer, of specific antibodies during the course of the illness

It is not always possible, nor necessary, to follow all three lines of attack in each case, the decision as to which approach should be followed is determined largely by the nature of the infection encountered, the stage of the illness in which the patient is first seen, and the amount of information that the method will yield in relation to the amount of time and effort involved.

Microscopic methods are generally simple, but they have a limited usefulness There are comparatively few clinical conditions in which this procedure can be used, for example, vaccinia and variola, but it is always well to confirm the findings by some other procedure such as serology or

TABLE 1

LABORATORY EQUIPMENT REQUIRED FOR VIRAL DIAGNOSTIC WORK

1 In vitro serology
   - Centrifuges, horizontal head
   - Drawers, with built-in racks, for serum storage, or test-tube racks for same purpose
   - Filters, Seitz, 2 ml size and up
   - Glassware—tubes, flasks, pipettes, etc
   - Pipetting machines
   - Plastic plates for hemagglutination tests or complement fixation
   - Racks, serologic
   - Refrigerator(s), 4° to 6° C, for sera, reagents, etc
   - Water bath, large, 37° C.
   - Water bath, medium size, variable temperature control, for agglutination tests
   - Water bath, small, temperature adjustable 56° to 65° C, for inactivation

2. Preparation of serologic antigens and for in vivo (neutralization) tests
   - Items in 1, above, plus
     - Animal boards
     - Animal cages
     - Bacteriologic incubator
     - Balances, trip, for weighing tissues
     - Deep-freeze box, electrical
     - Dental drill, for egg inoculation
     - Dry-ice chest
     - Egg candler
     - Egg incubator, 35° C (for incubation of inoculated eggs)
     - Egg incubator, 37° C (for incubation of stock eggs)
     - Glassware, assorted
     - Hoods, table-top, or safety cabinets
     - Hypodermic needles, assorted sizes
     - Microscope
     - Mortars and pestles
     - Surgical instruments (for use with animals and eggs)
     - Syringes, assorted sizes
     - Trocars, for egg inoculation
     - Waring blendors

3 Isolation of viral and rickettsial agents
   - Items in 1 and 2, above, plus
     - Animal cages, for various species of test animals
     - Animal isolation rooms
     - Analytical balance
     - Lyophile apparatus
     - Microtome, for histologic se
     - Safety cabinets
     - Spinco refrigerated, vacuum chamber, high speed centrifuge, with size No 30 and No 40 rotors
     - Ultraviolet lamp unit for sterilization of plastic centrifuge tubes

have an adequate knowledge of what material to take, how to take it, when to take it, and how to submit it for viral and rickettsial examinations. The proper collection, shipment, and processing of specimens is highly important to the success of any subsequent examination, and the full co-operation of the physician is necessary. This problem is an especially acute one in those laboratories whose services cover a wide geographic area.

In addition to well-chosen material taken at the appropriate time and shipped to the laboratory under proper conditions, a certain amount of information regarding both the patient and the specimens is required. It should be obvious, as Dudgeon points out, that "the pathologist needs a good clinical history to guide his choice of tests just as much as the clinician needs the laboratory findings to guide his diagnosis"[2] The submission of a good clinical history or a summary is highly desirable; lacking this, at least certain minimal details must be furnished before even a choice of a laboratory test can be made and before an intelligent and meaningful interpretation of the results can be made The minimal amount of information required may be obtained on a form such as that shown in Figure 1, which *should be included in every specimen-mailing* container issued by the laboratory

B. Collection of Material for Microscopic Examination

In making films or smears, clean slides should be used The material should be spread out as thinly as possible and allowed to dry Since diagnosis depends upon the presence of inclusion bodies or pathognomonic cells, or on actual demonstration of viral elementary bodies or of rickettsiae, it is important that the preparations contain tissue elements. In the case of cutaneous lesions—*for* example, variola, vaccinia, or herpes simplex—scraping of the lesions should be done with a scalpel, curette, or similar instrument and the minute fragments utilized Similarly, in the case of ocular lesions, smears should be made from material expressed from the follicles, smears made from cotton pledgets used to swab the conjunctival surfaces are seldom adequate The slides should be appropriately marked, and if they are to be shipped to a laboratory for examination, an indication should be given as to the type of condition from which the material was obtained in order that appropriate staining methods can be chosen. It should also be remembered that slides from cases of variola are infectious, and due warning should be given to the laboratory workers who are to handle such slides

Biopsy or post-mortem material for histologic examination should be placed in a fixative solution promptly after being taken While special fixatives are sometimes more satisfactory for specific purposes, Zenker's solution containing 10 per cent formalin is satisfactory for general purposes.

C. Collection and Handling of Blood Specimens for Serologic Tests

1. *Collection.* A serologic diagnosis is based upon the appearance or the increase in titer of humoral antibodies during the course of an illness. *Consequently at least two, and sometimes* more, specimens of blood taken during various stages of the illness are required. The first specimen of blood should be taken early in the acute phase of the illness or when a viral infection is suspected, this first specimen cannot be taken too early. In general the second specimen of blood should be taken from 10 to 14 days after the first. In some diseases, antibody is relatively late in appearing, and hence additional specimens are desirable One may be taken one month after the first and, if necessary, followed by additional specimens taken at later intervals as indicated by the clinical findings and the laboratory results.

It must be emphasized that serum for serologic tests in the viral and rickettsial *diseases must be taken under aseptic precautions and handled with aseptic techniques* from the time it is taken until it is finally discarded when all tests have been completed (sometimes weeks or perhaps months later) The use of nonsterile containers, nonsterile pipettes, and so forth, that is, the usual nonsterile technics employed by serologic laboratories, have no place in viral and rickettsial work. From 15 to 20 ml of blood should be taken with a sterile, dry syringe to avoid hemolysis No anticoagulants or preservatives should be used as these may interfere in the serologic tests

2 *Shipping and storage* If a specimen is to be sent to a laboratory at some distance, one or another course may be followed If facilities are available, the blood may be placed in a sterile tube and the serum later removed and transferred to a sterile, rubber-stoppered, or screw-capped vial for shipment As most physicians do not have the time or the facilities to separate the serum for shipment, whole blood may be sent to the laboratory, a procedure which may be encouraged, since it reduces difficulties caused by contaminated sera Transmittal of blood specimens is simplified by providing the physician with self-addressed mailing cartons containing sterile vials (*see Fig* 2) Information concerning the specimens should be sent with them The minimal essential information is shown in Figure 1, ideally, more complete information in the form of an abstract of the clinical and epidemiologic history of the patient should supplement the brief data submitted with the specimen

It is generally stated that serum should be removed from the clot shortly after the specimen is obtained, promptly frozen on dry ice, and shipped to the laboratory in the frozen state; in the laboratory, the specimens should be kept frozen by storage on dry ice or in a mechanically operated refrigerator at −10 to −20° C. However desirable such

a procedure may seem, there are a number of objections from the practical standpoint. First of all, few physicians have the facilities for properly processing serum, and insistence that every specimen be shipped on dry ice would place a considerable obstacle in the path of those seeking the assistance of the laboratory. From the standpoint of the diagnostic laboratory, there are also a number of objections, and these will be taken up further below. The main point of emphasis here is that if the physician is to utilize the diagnostic facilities made available to him, every effort should be made to simplify the task of collecting and shipping sera.

Whole blood should never be frozen since freezing will result in total hemolysis of the specimen. For the purposes of in vitro serology, there is no unequivocal evidence that shipment and storage of serum in the frozen state serves any special purpose nor is there in the published literature, so far as the author is aware, any conclusive or unequivocal evidence that preservation of sera in the frozen state is absolutely basic or mandatory for the proper performance of in vivo (neutralization) tests *in general*. (There appear to be certain exceptions, see discussion of neutralization tests.)

The reasons for storing sera in the frozen state are based on observations concerning two factors, namely, the presence of heat-labile, nonspecific neutralizing substances and the preservation of specific antibody. From the standpoint of the diagnostic laboratory, there is no serious objection to the storage of sera in the fluid state at 4° C.

3. *Handling of blood in the laboratory.* Blood should be allowed to clot at room temperature and, after retraction of the clot begins, the specimen may be held in the refrigerator until retraction is maximal, when the serum should be removed. Where the blood specimen is to be examined for the presence of cold hemagglutinins, it should be kept at room temperature or in an incubator at 37° C for 1 or 2 hours before removal of the serum in order to permit elution of the agglutinins from the red cells. Usually retraction of the clots has already occurred in whole blood specimens that have been shipped to the laboratory, and hence the serum can be removed promptly. Much time can be saved if the blood containers supplied by the laboratory fit standard centrifuge cups, otherwise, the serum must be removed with aseptic precautions, placed in another container, and centrifuged to deposit cellular elements. Avoidance of bacterial contamination is a problem even when sterile tubes or vials are supplied by the laboratory. Contaminating microorganisms may render the sera unsatisfactory or useless since the action of the contaminants results in anticomplementary effects, and when such sera are used for neutralization tests, the contaminants present may kill the test animals. Enzymatic action may also destroy antibody. Ideally, therefore, all sera should be routinely cultured on receipt, but if this is not feasible, bacteriologic

cultures should be made at least of those sera which have a suspicious appearance. As most contaminants are saprophytic organisms, relatively simple culture media such as glucose broth and sodium thioglycolate medium are adequate. Contaminated sera should be filtered through a Seitz pad, and small filters of 2 ml and 25 ml capacity are available for such purposes

As was mentioned above, anticoagulants should not be used in the collection of blood specimens as they may render the serum anticomplementary. Similarly, it is preferable to handle sera with aseptic technics than to rely upon preservatives Some preservatives may make the sera anticomplementary and, in addition, when the sera are also used for neutralization tests, the preservative may have a deleterious effect on the virus If it appears desirable to use a preservative for sera which will be used *primarily for in vitro serologic tests, a 1 50,000 concentration of phenyl* mercuric borate might be recommended, in practice, 1 drop of a 1·2,500 solution of the preservative is added per 1 ml of serum Phenyl mercuric borate and merthiolate have been found to have no deleterious effect on the viruses of Western equine and St Louis encephalitis when sera containing these preservatives were used in neutralization tests[3] Their effect on other viruses may be quite different, however Rogers,[4] for example, found that while merthiolate had no effect on the Eastern and Western equine viruses and the St Louis and Japanese B viruses, it did produce marked destruction of the viruses of lymphocytic choriomeningitis, encephalomyocarditis, and Colorado tick fever Preservatives, therefore, should be used with discretion

4. *Storing and filing specimens.* In the usual bacteriologic and serologic laboratory, sera are tested as they are received, and hence there is virtually no problem of storing and filing specimens. In the virus laboratory, the diagnostic procedures require examination of serial specimens. Hence provision must be made for the storage of individual specimens until all are received, and also there must be a filing system which permits instant location of any and all specimens on a given patient. One simple method is illustrated in Figure 3, which shows stainless steel units consisting of four drawers each and so made that the units can be stacked and the drawers freely interchanged. Wire partitions separate individual tubes, and the fixed spaces permit filing the tubes in numerical sequence by accession numbers which are assigned to specimens in order of their receipt in the laboratory.

Although diagnostic sera in our laboratory are stored, with few exceptions, at 4° C., the temperature at which specimens are to be held remains a decision for each laboratory to make on the basis of its own

specimens of serum from a patient are stored at 4° C., and then tested simultaneously, the test would show that serial specimens had progressively greater neutralizing capacities, the results would indicate that a rise in antibody titer had occurred during the illness, although the rise was spurious, that is, nonspecific. Morgan,[5] working with the Western equine encephalomyelitis virus, reported that the neutralizing capacity of sera stored at 4° C could be restored by the addition of fresh, unheated guinea pig serum, the addition of fresh guinea pig serum to sera which had lost some of their neutralizing capacity through heating at 56° C. could not restore the capacity Whitman,[6] working with the same virus, was able to restore the neutralizing capacity both of sera which had been stored and of sera which had been heated The work of Ginsberg and Horsfall [7] indicates that this neutralizing factor is a nonspecific substance present in many sera and that it can be destroyed by heating at 56° C for 20 to 30 minutes, a procedure which avoids complications arising from the presence of this factor It would therefore appear that one has his choice of avoiding difficulties associated with nonspecific neutralization by preserving this factor through storage of all sera in the frozen state or, if sera are stored in the fluid state at 4° C , by heating all specimens before testing Certain aspects of the problem are further considered under the section dealing with neutralization tests, a brief summary of literature on heat-labile neutralizing factors is given by McCarthy and Germer [8]

The effect of long storage at 4° C , or in the frozen state, on the antibody titer is essentially unknown, although there is some reason to believe that antibody titers, on the whole, are better maintained at subfreezing temperatures than those just above freezing The information available is derived from occasional rather small-scale, short-term studies, and it is desirable to obtain more definitive information through more extensive, well-designed, long-term studies which will permit a statistical evaluation of the findings

For storage at subfreezing temperatures, both glass and plastic containers have been used In the case of glass containers a tight screw cap or other type of closure is satisfactory at temperatures of -10 to -20° C , but if dry ice is used as the refrigerant, flame-sealed ampules must be used to protect the contents against the deleterious effects of carbon dioxide In either case, there is the problem of breakage, and to avoid this, plastic tubes have been used The objection to this type of container is not only that carbon dioxide penetrates the contents regardless of the type of closure used, but also the mouth of the tube cannot be flamed, and the risk of contamination is undesirably increased In either case, whether glass or

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[illegible]

of storage at 4° C. but is preserved by freezing. Consequently, if multiple

(or tissue cultures) it should be taken with sterile precautions so as to avoid contamination of such material as cerebrospinal fluid or biopsy tissue, which is initially sterile, or to add further contamination to material already containing bacteria; for example, throat washings or lesion scrapings. Post-mortem material is usually taken by nonsterile technics, but should nevertheless be handled in the laboratory with sterile technics to avoid further contamination Stools, or rectal swabs, are, of course, heavily contaminated, but should also be handled with sterile precautions for the sake of maintaining good technic, avoiding cross-contamination of specimens and protecting laboratory personnel Fluid materials such as throat washings or cerebrospinal fluid and solid materials such as stools or tissues should be placed in sterile containers, labeled, and placed under refrigeration immediately To prevent desiccation, swabs should be placed in a tube containing 0 5 ml or so of nutrient broth or other suitable diluent Vesicle fluid is collected in small capillary tubes whose ends may be closed with beeswax or a similar substance Like other materials for virus isolation work, these specimens should be immediately refrigerated and should be kept cold during shipment and until finally inoculated into the appropriate animals

3 *Shipment* If shipment to a laboratory at some distance is required, dry-ice refrigeration should be employed Although thermos jars are often used as shipping containers, their use is not recommended because of the ease with which they are broken in transit Containers made of fiberboard or plastic, which are lightweight, well insulated, durable, and resistant to breakage, are available commercially * Whether such containers, or ordinary cardboard cartons, are used for shipment, the following procedure is recommended The tube or jar holding the specimen should be placed in a container, preferably a metal one, with sufficient crumpled paper or cotton to act as a shock absorber and to take up any fluid should breakage occur during transport This metal container (or heavy cardboard container if used) should then be placed in a shipping container such as that just mentioned above or in a larger cardboard carton into which can be placed sufficient dry ice to keep the specimen frozen for the period required to reach its destination With the dry ice there is included sufficient shredded paper, excelsior, wood shavings, or vermiculite to take up the space released by evaporation of the dry ice and to act as a protective shock absorber to the smaller

* One type, made of styrofoam, for example is manufactured by the Hollinger Corporation, 3834 South Four Mile Run Drive, Arlington 6 Virginia

plastic containers are used, the material should be distributed into several aliquots This protects against complete loss of the specimen, should a glass container break, and also against the deleterious effects believed to occur with repeated freezing and thawing of a specimen.

D Collection and Handling of Specimens for Virus Isolation

The usual types of material collected for virus isolation are blood, throat washings, cerebrospinal fluid, stools, effusion fluid, vesicle fluids, lesion scrapings, biopsy tissues, and post-mortem tissues. The type of material to be collected depends upon the nature of the illness, as is indicated in Table 2 Thus, in upper respiratory diseases, throat garglings are taken; sputum or effusion fluid, as the case might be, in instances *of pulmonary involvement*, *cerebrospinal fluid in central nervous system* disturbances, blood in rickettsial diseases, and so on. This applies primarily to those cases in which the clinical picture and epidemiologic history give some clue to the possible etiologic agent, or agent group, involved In those obscure infections which represent a diagnostic riddle, a decision as to the type of material to collect is not always simple to reach In essence, the patient represents a research problem, and the *presence of the agent must be sought for in a variety of materials.* Obviously, even a large laboratory cannot handle many such problems because of the cost in terms of money and personnel. Nevertheless these represent important clinical problems whose resolution may contribute to our knowledge of viral diseases

1 *Time of collection* Proper collection and handling of the specimen is highly important to the successful recovery of any agent which might be present In general, the material should be collected as early as possible in the acute stage since the agents tend to disappear relatively rapidly after the onset of illness Thus, influenza virus may be recoverable from throat washings taken during the first 2 to 3 days of the illness, but the chances of successful recovery are greatly lessened thereafter Similarly, in the rickettsial diseases a rickettsemia occurs for only a few days after the onset of symptoms The poliomyelitis and Coxsackie viruses, on the other hand, may persist in the stools for many days, but again it is preferable to obtain specimens for isolation purposes early in the acute phase of the illness, if attempts are made later in the illness, a series of specimens should be examined.

2 *Collection.* Since the material is to be inoculated into animals

St. Louis encephalitis	?[c]		?[c]		?[c]					+	Mouse, i.c.; chick embryo, intra-amniotic
Venezuelan equine encephalomyelitis	+		+		+					+	Mouse, i.c.; chick embryo, intra-amniotic
Western equine encephalomyelitis	?[c]		+		?[c]					+	Chick embryo, intra-amniotic; mouse, i.c.
Skin and mucous membrane infections											
Herpangina (Coxsackie virus)	+			+							Suckling mouse, i.c. and i.p. combined
Herpes simplex	+					+[d]	+				Suckling mouse, i.c. and i.p. combined, chick embryo, C-A membrane
Vaccinia							+		+[e]		Chick embryo, C-A membrane
Variola	+		+				+		+[e]		Chick embryo, C-A membrane
Miscellaneous viral infections											
Colorado tick fever			+								Suckling mouse, i.c. and i.p. combined
Dengue			+								Suckling mouse, i.c. and i.p. combined
Lymphogranuloma venereum						+[d]	+				Mouse, i.c., chick embryo, yolk sac
Mumps	+										Chick embryo, intra-amniotic
Newcastle disease						+[d]					Chick embryo, intra-amniotic
Rickettsial group											
The typhus fevers, spotted fever, Q fever, rickettsialpox, etc.			+						+		Chick embryo, yolk sac. Animal species according to epidemiologic and clinical history of patient, or to type of agent endemic in area—see chapter on rickettsial diseases

[a] Use heparinized blood or blood clot.
[b] Also pleural fluid, if available.
[c] Saliva.
[c] Virus rarely encountered or presence unknown so far.
[d] In ocular infections.
[e] As well as material listed under "clinical."

TABLE 2

SOURCE MATERIAL UTILIZED IN DIAGNOSIS BY METHOD OF VIRUS ISOLATION

Disease Suspected	Clinical Material: Throat Washings	Sputum	Blood*	Feces	CSF	Eye Washings, Conjunctival Scrapings	Local Lesions Fluid, Pus, Crusts, Scrapings	Post-Mortem Material: Lung	Liver, Spleen	CNS	Test Species and Route of Inoculation
Respiratory infections											
Epidemic pleurodynia (Coxsackie virus)	+			+							Suckling mouse, i c. and i p combined
Influenza A, B, and C	+							+			Chick embryo, intra-amniotic
Ornithosis	+	+[a]	+					+	+		Chick embryo, yolk sac, mouse, intracerebral
"Viral pneumonia"	+	+[a]						+			Chick embryo and mouse, use several routes, *tissue culture for adeno*
C N S infections											
Herpes simplex	+				+					+	Suckling mouse, i c and i p combined, chick embryo, C-A membrane
Lymphocytic choriomeningitis			+		+					+	Mouse, i c., guinea pig, i c.
Lymphogranuloma venereum					+				+	+	Mouse, i c, chick embryo, yolk sac
Mumps meningoencephalitis,	+				+					+	Chick embryo, intra-amniotic
Poliomyelitis	+			+						+	Tissue culture
Rabies	+[b]									+	Mouse, i c.
Eastern equine encephalomyelitis	?[c]		?[c]		?[c]					+	Mouse, i c.; chick embryo, intra amnio
Japanese B encephalitis	?[c]		?[c]		?[c]					+	Mouse, i c.; chick embryo, intra-amnio

TABLE 3

DISEASES IN WHICH MICROSCOPIC METHODS ARE AIDS IN DIAGNOSIS

Disease	Method		Material	Remarks
	Smear	Histologic Sections		
Rabies	+	+	Brain	Virus isolation desirable
Yellow fever		+	Liver	Virus isolation desirable
Ornithosis	+	+	Avian spleen, air sac	Serologic tests and/or virus isolation desirable
Lymphogranuloma venereum	+	+	Buboes, affected tissue	Serologic tests and/or virus isolation desirable
Vaccinia	+	+	Scrapings from lesions	Serologic tests and/or virus isolation desirable
Variola	+	+	Scrapings from lesions	Serologic tests and/or virus isolation desirable
Herpes simplex	+	+	Scrapings from lesions	Serologic tests and/or virus isolation desirable
Varicella	+		Scrapings from lesions	Differentiation from vaccinia-variola and herpes simplex requires additional methods See appropriate chapters
Herpes zoster	+		Scrapings from lesions	
Trachoma	+		Epithelial scrapings, follicular expressions	
Inclusion blennorrhea	+		Epithelial scrapings, follicular expressions	
Molluscum contagiosum	+	+	Exudate from nodule, biopsy material	
Verruca vulgaris		+	Biopsy material	

unit holding the specimen itself. In accordance with postal regulations every precaution must be taken to afford protection to those handling the material in transit.

If dry ice is not available, recourse may be had to glycerin. In such case, the glycerol should be of a good brand, of high quality, neutral, and used as a 50 per cent solution in buffered saline (pH 7.4)

4 *Storage*. In the laboratory the specimens are preferably stored on dry ice. While a storage temperature of —20° C. to —25° C. is adequate for the preservation of many viruses, the infectivity of others diminishes appreciably at these temperatures within a matter of a few months.[9,10] On dry ice the infective material can be preserved for months and even years if necessary It is well to keep the specimens in flame-sealed ampules until all the tests have been completed in order to avoid the deleterious effects of CO_2 on the viability of the agents. Figure 4 illustrates a cabinet of the type devised by Horsfall [11,12] for storage of materials on dry ice. The drawers are designed to hold small, soft-glass, shell-type ampules and also to provide for the storage of material in larger containers, such as flame-sealed test tubes and wide-mouthed jars of various sizes. Figure 5 illustrates a method for the storage of specimens in compartmented drawers built to fit into an electrically operated, upright, deep-freeze unit. Each drawer slides out on fixed guide rails, and the size of the compartments in each can be altered as desired by means of removable dividers held in slots.

V. SOME GENERAL CONSIDERATIONS OF THE SEVERAL DIAGNOSTIC APPROACHES

A. Microscopic Methods

Under this designation is included the examination of fixed and stained tissues for the presence of pathologic or pathognomonic changes, as well as the examination of imprint preparations or smears from tissues, fluids, exudates, and so on, for the presence of inclusion bodies, abnormal cells, and even the agent itself.

A list of conditions in which direct examination of tissues or other materials is helpful in diagnosis is given in Table 3 Full descriptions [illegible] in the [illegible] ecif- [illegible] com- prehensive array of useful staining procedures may be found in the large

occurs in the vaccinated person is not always readily accomplished on clinical grounds alone. Establishment of a definitive diagnosis is extremely important to the public health officer since it determines whether an elaborate mechanism for prevention and control is to be set in motion, and the aid of the laboratory is frequently enlisted. Stained smears revealing characteristic inclusions prepared from papules and from vesicle fluid are valuable in arriving at an early presumptive diagnosis, findings with material containing pus are difficult to evaluate because of the obscuring debris Correct interpretation requires experience and must always be evaluated in the light of clinical and epidemiologic data Confirmation must be sought by other means, either isolation of the agent in embryonated eggs or use of the vesicular fluid as an antigen in the complement fixation test Other larger viruses and the rickettsiae may often be visualized by direct examination At times elementary bodies may be seen in the pus from the buboes of lymphogranuloma venereum Other agents of the psittacosis-lymphogranuloma venereum group, as well as rickettsiae, are not often present in large enough numbers in human tissues to be detected, the organisms may, however, frequently be seen in the tissues of naturally infected animals, and usually, in the tissues of inoculated animals. Elementary bodies may be present in smears prepared from the enlarged spleen or the air sac of birds suspected of ornithosis Similarly, the rickettsia of Q fever may be seen in the cotyledons of the placenta of an infected sheep.

Smears of epithelial scrapings or follicular expressions from cases of trachoma or inclusion blennorrhea generally contain the pathognomonic inclusion bodies which are readily seen under the microscope

B Isolation of Viruses

As will be described later, there are conditions under which isolation of the virus is a desirable procedure or the procedure of choice. However, for routine diagnostic purposes, virus recovery is so laborious, time-consuming, and costly a procedure that it is avoided wherever diagnosis can be made by serologic means

Of the conditions under which isolation of the agent is desirable, one has already been mentioned, namely, the need for confirmation of interpretations based on microscopic methods Also, attempts at recovery of an agent are virtually mandatory in attempting to establish the etiology in obscure or vague conditions of presumed viral causation In addition, virus isolation attempts are frequently desirable in attempting to resolve the problems confronting a health officer in the presence of an outbreak of disease of unknown etiology The latter two situations

text of van Rooyen and Rhodes[13] and also in the excellent smaller work by these same authors.[14]

Histopathologic methods have, on the whole, a limited application since their value in establishing an etiologic diagnosis is limited to a few diseases In the encephalitides, for example, histopathologic methods are of no value in establishing an etiologic diagnosis, although they may serve to differentiate an infectious process from a noninfectious one, or a necrotizing process from the demyelinating types seen in the post-vaccinal type of encephalitis Other examples might be cited, but it is for such reasons that the pathologist, in addition to securing material for gross and microscopic examination, should also set aside tissues which might later be used for virus isolation attempts if the results of the microscopy point to the desirability of such a procedure. Of those instances in which histopathologic methods serve to identify the causal agent, perhaps the most obvious examples are the examination of brain tissue for the presence of Negri bodies and the examination of liver tissue obtained at post-mortem or by viscerotomy for the presence of changes produced by the yellow fever virus In the virus laboratory, histologic methods probably find the greatest usefulness when applied to the examination of tissues from animals used in virus isolation work; for example the Coxsackie group of viruses In this instance, examination of brain, muscle, and pancreas permits rough classification of the agent as to group, for more precise classification, the agent must be typed by serologic methods.

Microscopic examination of imprint preparation or smears is far less time-consuming and laborious than is the preparation of tissue sections and hence finds a somewhat wider application than do histopathologic methods As in histopathologic methods, the conclusions reached by direct examination should be supported or confirmed by another method, usually isolation of the virus The need for confirmation holds even though the findings are evaluated, as they always should be, on the basis of the patient's clinical course and his epidemiologic background For example, in the case of rabies, errors in interpretation can occur even in experienced hands and, in addition, occasional preparations may apparently contain no Negri bodies although the virus is present in the tissue; consequently, it is well to confirm all interpretations, whether positive or negative, through routine inoculation of the specimens into animals for virus isolation

Differentiation of variola from varicella or its recognition when it

occurs in the vaccinated person is not always readily accomplished on clinical grounds alone. Establishment of a definitive diagnosis is extremely important to the public health officer since it determines whether an elaborate mechanism for prevention and control is to be set in motion, and the aid of the laboratory is frequently enlisted Stained smears revealing characteristic inclusions prepared from papules and from vesicle fluid are valuable in arriving at an early presumptive diagnosis, findings with material containing pus are difficult to evaluate because of the obscuring debris Correct interpretation requires experience and must always be evaluated in the light of clinical and epidemiologic data. Confirmation must be sought by other means, either isolation of the agent in embryonated eggs or use of the vesicular fluid as an antigen in the complement fixation test Other larger viruses and the rickettsiae may often be visualized by direct examination At times elementary bodies may be seen in the pus from the buboes of lymphogranuloma venereum. Other agents of the psittacosis-lymphogranuloma venereum group, as well as rickettsiae, are not often present in large enough numbers in human tissues to be detected, the organisms may, however, frequently be seen in the tissues of naturally infected animals, and usually, in the tissues of inoculated animals Elementary bodies may be present in smears prepared from the enlarged spleen or the air sac of birds suspected of ornithosis Similarly, the rickettsia of Q fever may be seen in the cotyledons of the placenta of an infected sheep

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represent, in essence, research problems, and while the main function of a virus diagnostic laboratory is to provide facilities for *routine* diagnosis, the laboratory should also try to participate, insofar as its facilities permit, in the investigation of disease

In addition to the above situations, there are others in which isolation of the virus is essential in establishing a diagnosis or in differentiating clinically similar conditions The viruses of variola and vaccinia, for example, cannot be readily distinguished by serologic methods, and differential diagnosis between variola and generalized vaccinia requires isolation and identification of the causal agent. Similarly, in herpetic eruptions, differentiation between herpes simplex and herpes zoster may, especially in individuals who have had recurrent attacks of herpes simplex, require recovery of the herpes simplex virus

In attempts to isolate a virus, recovery of an agent represents a significant finding but negative results do not necessarily exclude an agent suspected to be etiologically responsible for the illness in question Conversely, the mere recovery of an agent does not necessarily establish that it is responsible for the patient's current illness, the significance of a positive finding must be evaluated in terms of the patient's clinical picture and what is known of the natural history of the recovered agent Some agents may persist in the body for weeks, months, or even years after they have produced infection, either frank or inapparent Thus, recovery of herpes simplex virus from the mouth or throat of an individual with an obscure or indefinable illness does not prove that this virus is the causal agent Similarly, mere recovery of poliomyelitis virus from the stools of an individual with a vague illness does not establish a diagnosis of poliomyelitis, the illness may be totally unrelated, and the individual is experiencing an inapparent infection with the virus The same may be true of the Coxsackie viruses

Similar need for evaluation exists, for example, in those areas where two diseases are present endemically and occur at the same time In California, by way of illustration, Western equine encephalomyelitis and St Louis encephalitis occur during the same time of the year that poliomyelitis is highly prevalent It is thus possible for an individual to have a clinical infection with one of these viruses and a subclinical or inapparent infection with another If only serologic tests were done for the arthropod-borne encephalitides, a rising titer of antibody to the Western equine virus might be found and the infection considered to be due to this agent Recovery of a poliomyelitis virus from the stool would contribute little to resolution of the diagnosis, and an evaluation of this finding would be required in terms of whether a rise in specific antibody to the type of poliomyelitis virus isolated had occurred, the nature of the symptoms, etc

A similar situation obtains insofar as the poliomyelitis and Coxsackie group of viruses is concerned The Coxsackie viruses are ubiquitous, and their role in the causation of human illness, except for herpangina and epidemic pleurodynia (Coxsackie Group A and B viruses, respectively), is not fully known There are instances of clinically typical poliomyelitis in which only a Coxsackie virus has been recovered and a rise in antibody titer to this virus demonstrated in the patient With the development of simpler in vivo and in vitro technics for isolation of poliomyelitis virus and for assay of poliomyelitis antibody, and their application to

such cases, there will be greater opportunity to determine whether the poliomyelitis viruses or the Coxsackie viruses are the inciting agents in such instances There is no good evidence at the present time to incriminate the Coxsackie viruses generally as incitants of CNS disease, and this illustration is provided as an example that even the recovery of an agent and the demonstration of a rise in antibody titer to it are not necessarily adequate to establish its causal relationship to a clinical case The laboratory findings must be compatible with the clinical and epidemiologic features which experience over many years has shown to be associated with a given agent

If what is apparently a new type of virus is isolated, the findings must be accepted with reservations until it is proved that the agent was actually isolated from the patient and does not represent a latent or wild virus of the test animal host, laboratory animals are not infrequently infected with viruses common to their species, and these latent agents may be activated by mere passage of the virus-containing tissues of the test animals This is a possible pitfall which should always be kept in mind

If, on the other hand, attempts at virus isolation are negative, it is possible that one or another of the criteria for successful recovery of an agent has not been met These criteria are

1 Collection of the clinical or post-mortem material most likely to contain the virus (see Table 2)
2. Collection of the appropriate material at the proper stage of the illness (see Table 4)
3 Proper handling of the material immediately after collection and during shipment to the laboratory (see section on collection and handling of materials, above)
4 Selection of appropriate, that is, susceptible, laboratory test hosts

1 *Collection of material* As to the first requisite for successful isolation of an agent, namely, collection of the material most likely to harbor the agent, Table 2 lists the sources found most frequently to contain the causal agent of some of the more commonly encountered viral and rickettsial diseases The type of material to be collected is determined by the nature of the illness, clinical suspicion as to the nature of the agent involved, and at least some knowledge of the pathogenesis of specific viral and rickettsial diseases In addition to selecting the appropriate material for examination, it is also necessary that it be taken at the optimal time during the early acute phase of the illness, the sooner after onset, the better, since the opportunities for successful isolation diminish rapidly with time, when the rallying defense mechanisms and antibody formation begin to exert an increasingly greater influence on the course of the disease This relationship is illustrated in Table 4, which has been modified after Horsfall[15]

2 *Selection of laboratory host* Assuming that the proper material has been taken at the proper time, one must then handle it as outlined previously to ensure the viability of any agent present In the laboratory, inoculation of the material into a susceptible host constitutes an important requirement

TABLE 4

RELATION OF STAGE OF ILLNESS TO PRESENCE OF VIRUS IN TEST MATERIAL AND TO APPEARANCE OF ANTIBODY

Stage of Illness	Virus Demonstrable in Appropriate Test Material	Specific Antibody Present in Serum
Incubation period	Rarely	
Prodromal period	Rarely	
Onset	Frequently	
Acute phase	Frequently	Frequently or generally*
Recovery phase	Rarely	Generally
Convalescence	Very rarely	Usually

Adapted from Horsfall [15]

* In certain widespread, endemic diseases, antibody representing prior experience with the agent is generally encountered in acute-phase blood (e g , influenza, herpes simplex) In other instances (e g , Western equine encephalitis, poliomyelitis) antibody is frequently present in acute-phase serum, antibody formation apparently is well under way by the time the acute-phase specimen is taken

The question of suitable laboratory hosts, of the methods and technics for viral isolation, requires discussion from the standpoint of whether the laboratory doing the diagnostic work is concerned primarily with diagnosis in the everyday application to clinical medicine or primarily with "diagnosis" as applied to research problems or epidemiologic investigations In the routine diagnostic laboratory, the use of a laboratory host is governed by its availability and its cost, the latter item including the amount of personnel time involved in carrying out the procedure In view of the fact that routine diagnostic laboratories have a very limited budget, usually a very small staff, and also must be in a position to undertake examinations for a large variety of diseases, unusual or costly host systems as well as cumbersome, time-consuming, intricate, or capricious methodologies are not feasible.

Thus, while monkeys, until the recent advent of tissue culture methods, were virtually the only susceptible laboratory host used for the isolation and identification of poliomyelitis viruses in investigative work, the cost precluded their use in applied diagnosis Similarly, the

ferret, once widely used for the isolation of influenza viruses and some years ago replaced by the embryonated egg, would have found a relatively restricted application in the diagnostic laboratory because of its cost as well as of the need for strict isolation of each inoculated animal

Guinea pigs, rabbits, hamsters, and occasionally other species have a role in specific examinations but are not so widely used as the mouse and the chick embryo, which are susceptible to a wide variety of agents.

Susceptibility to an agent depends not only upon the species but also upon the age of the test animal.[1,16] It is now well established that immature mice may be highly susceptible to infection with an agent to which the adult mouse may be completely resistant, and it will be remembered that the use of new-born mice has served to uncover the Coxsackie group of viruses, a whole new group of agents pathogenic with few exceptions only for very immature animals. Yet, it is possible that an agent may grow better in the mature than in the young animal as, for example, is true of the Lansing strain of poliomyelitis virus, although this strain admittedly has had a long period of adaptation to the adult mouse. Because of such considerations, it is advisable to employ both new-born and adult mice for virus isolation purposes. This also takes into account the fact that newly isolated viruses do not always behave according to the description of pathogenic properties given in textbooks,[17] which are commonly based on experimental work employing highly adapted laboratory strains.[1]

The value of the developing chick embryo in virology has recently been reviewed in an excellent article by Cox.[18] As is indicated in Table 2, the embryonated egg and the mouse comprise the two most useful laboratory test animals While various routes of inoculation are used for special purposes, the amniotic and the yolk sac routes probably are those most commonly employed for isolation work. There is a variety of technics for inoculation, harvesting, and passaging, but those who engage in large-scale diagnostic work will find that the simplest procedures are the most useful The window technic for exposure of the chorioallantoic membrane or similar exposure of the allantoic or amniotic cavities requires time Consequently, our standard operating procedure consists of direct inoculation of the chosen site without any preliminary exposure Direct exposure is done only occasionally and then for special purposes, as plaque counts. Similarly, time is not taken to harvest and process the whole embryo, or part of an embryo, when the fluids alone will suffice

Like other laboratory animals, the chick embryo may contain endogenous agents of its own. According to Cottral,[19] there is evidence that certain viral and bacterial agents affecting poultry are transmitted through the egg. In addition to these poultry pathogens, bacteria of many other types have been isolated from eggs. While it is theoretically possible that endogenous viruses might be carried along in serial passage, either alone or in combination with a human or animal virus one is trying to isolate, there is no good evidence that such occurs; in all probability these pathogens kill the embryos very early. As to the bacterial agents, most of these are detected by routine bacteriologic cultures made on passage material, and they are important chiefly as the cause of embryo mortality.

3. *Evidence of infection.* If the material under examination contains a viral or rickettsial agent, evidence of infection in the test animal is recognized in one of several ways, depending upon the test species and the pathogenic properties of the agent (see Table 5).

Where overt signs are produced as, for example, in the mouse inoculated with a neurotropic agent, it is sometimes possible to identify the virus presumptively on that basis (ruffled fur, hunched back, tremors, ataxia, spastic or flaccid paralysis, convulsions, etc.) and knowledge of the patient's clinical and epidemiologic history. Since the symptomatology is not distinctive for each type of agent, it serves more commonly only to give an indication as to the group of agents with which one is dealing. Sometimes the infectivity of the test material is so low that only minimal signs of infection are produced; the trained observer will note that one or more of the test animals in the group does not appear quite normal. Such suspected animals should be killed and further passages made. In other cases, the animals may appear definitely ill but not to the extent that death may be expected to ensue; such animals should be killed, the organs examined for the presence of lesions (for example, in the lungs after nasal instillation of throat washings from suspected *influenza*), or for the presence of the agent (for example, smears of the enlarged spleen in mice inoculated with suspected psittacotic material), and additional passages made. In still other instances, no signs of infection may appear, and infection is detectable only through examination of the serum of the test animals for antibodies (for example, hamsters inoculated with material containing *Coxiella burnetii*).

Similarly, in the chick embryo, virus multiplication may or may not cause the death of the embryo; on the whole, fatal infections of the embryo are less common than they are in a species such as the mouse. Where survival occurs, the presence of virus is detectable only by supplemental tests (Table 5). In the case of the rickettsiae and the larger viruses, microscopic examination of the yolk sac mem- [illegible] influenza,

[illegible] agglutinins

[illegible] the fluids

Failure to isolate a virus may be due to the fact that no agent was present in the original material or that the requirements mentioned elsewhere above were not properly fulfilled. Even when these requirements have been fully complied with, however, failure to recover an agent may be due to its presence in the test material in concentrations too low for detection, or it may be "masked," that is, rendered noninfectious by antibodies present in the specimen.

4. *Tissue culture.* The recent development by Enders and his associates[20,21] of a tissue culture technic of the "fixed cell" type for the propagation of poliomyelitis virus represents an important and an outstanding contribution. Tissue culture technics are described elsewhere in this book and represent, in large part, simplifications of the original procedures. Those undertaking the use of this technic for routine diagnosis should recognize that, although not intricate, the method is influenced by so many intangible and indefinable factors that its mastery to the point where it can be conducted smoothly and without difficulties requires time. Nevertheless the technic is such an important addition to the diagnostic armamentarium that its inclusion in the operational procedures of a diagnostic laboratory is to be encouraged.

While human embryonic tissue has been almost entirely replaced by monkey tissue (testicular and renal) as the cellular component for the cultivation of poliomyelitis and other viruses, human tissue is still highly useful where it is readily available and the cost of monkeys is a factor. The newer technic, however, employing the HeLa strain of human carcinomatous cells[22] possesses the virtue that these cells are readily cultivable by transplant and thus permit maintenance of a tissue source from which cultures can be prepared as required. The full capabilities of the new tissue culture technics have yet to be assessed, and the limitations delineated, hence, little can be said on the subject of criteria which provide evidence that multiplication of an agent is occurring. When a cytopathic effect is produced, as in the poliomyelitis viruses, direct evidence of viral multiplication is at hand, it is necessary, however, to rule out nonspecific degeneration such as that due to toxic substances in the nutrient fluid or in the inoculum or bacterial contamination. It appears likely that under some conditions, that is, where an agent exerts no cytopathic action, evidence of infection may rest upon demonstration of the appearance of complement-fixing antigens or hemagglutinins.

C. Serologic Methods

By far the greatest proportion of examinations conducted in a diagnostic laboratory consists of serologic tests. These comprise the complement fixation, agglutination, hemagglutination-inhibition, and in vitro neutralization technics. The complement fixation method finds the greatest application, with hemagglutination and neutralization tests next, and

TABLE 5

Evidence of Infection in Laboratory Hosts Inoculated with Clinical or Post-Mortem Material Containing Virus

Virus Present in *Test Material*	Test Host Used	Route of Inoculation	Evidence of Infection
Influenza	Chick embryo	Intra amniotic or intra allantoic	hemagglutinins
Mumps	Chick embryo	Intra-amniotic	hemagglutinins
Newcastle disease	Chick embryo	*Intra-amniotic*	hemagglutinins
Variola	*Chick embryo*	Chorioallantoic membrane	*tance of complement*-fixing antigen and
Psittacosis	*Chick* embryo	Intra-allantoic or yolk sac	*death of embryo, elementary bodies in* *r in lining cells of yolk sac*
	Mouse	Intra-abdominal	s death, with elementary bodies udate, although subpassages *may* be survival—challenge inoculation with t psittacosis virus required
Lymphogranuloma venereum	Chick embryo	Yolk sac	*o, elementary bodies* in lining cells of
	Mouse	Intracerebral	ht loss, *hunched gait* *Impression* ral cortex and meninges show elementary
Yellow fever	*Monkey*	Subcutaneous *or* intra-abdominal	val: blood taken during *febrile episode,* *mice* for recovery of virus opic lesions in liver (not always *reliable*— s above)
	Mouse	*Intracerebral*	ching, etc., death
Dengue fever	Mouse (1-3 days old)	*Intracerebral*	a, death If all mice survive, challenge essary
Rift Valley fever	Mouse	Any route	hepatic lesions and intranuclear inclusions
Colorado tick fever	*Mouse (1-3 days old)*	Intra-abdominal	n
erpes simplex	Chick embryo	Chorioallantoic membrane	
	Rabbit	Corneal scarification	vitis
ymphocytic choriomeningitis	Mouse	Intracerebral	sions, generalized rigidity, death
	Guinea pig	Intracerebral	mors, *ruffled fur*, death
abies	Mouse	Intracerebral	rs, inco-ordination, paralysis, convulsions. brain

iomyelitis	Tissue culture		n of cells (cytopathogenic effect) Appearance ment-fixing antigen
	Monkey	Intracerebral	alysis of limbs (Destruction of anterior horn inal cord)
Coxsackie viruses	Mouse (1-3 days old)	Intracerebral	tremors, ataxia, spasticity or flaccid paralysis death Generalized or focal myopathy; lesions eart, liver, pancreas
Eastern equine encephalomyelitis	Chick embryo	Intra-amniotic	pearance of complement fixing antigen
	Mouse	Intracerebral	, tremors, ataxia, etc , death
Western equine encephalomyelitis	Chick embryo	Intra amniotic	pearance of complement fixing antigen
	Mouse	Intracerebral	, ataxia, etc , death
Venezuelan equine encephalomyelitis	Chick embryo	Any route	pearance of complement-fixing antigen
	Mouse	Any route	taxia, ruffled fur, convulsions, death
St. Louis encephalitis	Mouse	Intracerebral	ability, ruffled fur, convulsions, etc ; death
Japanese B encephalitis	Mouse	Intracerebral	taxia, etc , death
Epidemic typhus fever	Guinea pig	Intra-abdominal	Tunica reaction (male pigs) may occur ce of complement-fixing antibody
	Cotton rat (*Sigmodon hispidus hispidus*)	Intracardial	n dose rickettsiae is massive Survivors *omplement fixing antibody* and resist challenge n
	Chick embryo	Yolk sac	in impression smears of yolk sac membrane ed embryos
Endemic (murine) typhus fever	Guinea pig	Intra-abdominal	ica reaction (male pigs) frequent Complement- ibody appears in survivors
Scrub typhus	Mouse	Intra-abdominal	ofibrinous peritonitis and enlarged spleen Rickettsiae in smears from surface spleen and eritoneum
	Chick embryo	Yolk sac	mbryo without pathognomonic lesions ie present in smears of yolk sac lining cells es blind passages may be required)
Q fever	Guinea pig or hamster	Intra abdominal	iths not usual Complement-fixing antibody i blood
	Chick embryo	Yolk sac	ariable proportion of embryos Rickettsiae in s of yolk sac
Rocky Mountain spotted fever	Guinea pig	Intra-abdominal	ere scrotal reactions in male pigs Develop- omplement-fixing antibody and resistance to with *R rickettsii*

the agglutination test the least commonly used Table 6 presents a list of common diseases in which serologic tests are applicable and the types of tests used

1 *Complement fixation tests.* The complement fixation test represents a simple and highly satisfactory method for the examination of large volumes of material There is a variety of technics, the differences lying in the concentration of reagents used, the volumes of each employed, the time and temperature of the primary incubation period, and the length of the secondary incubation period before readings are made. The variations which have arisen over the past years are derived in part from efforts to avoid difficulties engendered by impure and insensitive antigens and in part to personal preferences developed through individual experience. There is no real need for many of these differences today since very good, and even excellent, antigens are available for many of the diseases for which tests are conducted The differences in many cases are minor, yet the reader, in going through the literature, may well come to the conclusion that there is a different method for each viral disease. The development of a single standard test acceptable to all investigators is an ideal yet to be achieved. In the interim, however, those undertaking diagnostic work are advised to select one complement fixation method and, with the objective of applying it to as many viral and rickettsial diseases as possible, modify it accordingly to provide a single, basic test This suggestion is based on at least two practical considerations, first, that if the volume of material to be handled is large, examination is greatly simplified and expedited by a single "standard" method, and second, the possibility of errors is greatly reduced when examinations are conducted by a single procedure, that is, the greater the number of variations of a technic employed, the greater the opportunity for error.

In selecting a complement fixation method or in adapting it to specific purposes, sensitivity and specificity are paramount considerations These two factors are to some extent inversely related so that a gain in one property leads to a concomitant loss in the other. This is illustrated in a recent evaluation of several complement fixation technics for the laboratory diagnosis of the rickettsioses;[23] comparison of 5 different technics showed that the most sensitive method of the 5 was the least specific and that the least sensitive method was the most specific, with the other 3 procedures falling in between A highly sensitive test may be desirable for certain purposes such as serologic surveys

to determine the extent to which immunity to a particular agent is present in a population, however, the degree of nonspecificity associated with an oversensitive test may well give rise to misleading findings if appropriate and adequate measures are not taken to detect these spurious positives In diagnostic work, tests with the highest degree of sensitivity, compatible with a high degree of specificity, are a desideratum if the number of false positive reactions is to be kept at a minimum and the number of missed diagnoses is also to be minimal

For those desiring to apply a single method to the diagnosis of a variety of viral and rickettsial diseases, the modified Kolmer technic may be recommended on two counts (1) it "occupies a happy middle position of balance between sensitivity and specificity,"[23] and (2) it is already familiar to most workers in public health laboratories. Another procedure which is essentially a modified Kolmer technic is the standard test promulgated by the World Health Organization for Q fever studies.[24] The test used in our laboratory differs in some respects from the Kolmer technic but primarily in the use of smaller volumes of reagents in order to conserve serum for the battery of tests that is frequently required for diagnosis

Sensitivity and specificity are affected by the accuracy with which reagents are standardized in relation to each other Precision in measurement is especially important when small volumes are employed. The need for the utmost accuracy becomes obvious if it is recalled that the complement fixation test consists, in essence, of 5 variables, 3 of which —antigen, complement, and hemolysin—are assayed for use in the test on the basis of a 4th variable, sheep erythrocytes, which are used directly in the test The final step is titration of the 5th variable—the serum under examination Consequently, the test is open, as is any system of variables, to an over-all error greater than the expected error due to each of the variables[25] Failure to recognize this leads to results which cannot be replicated Wide variation in the results on replicate testing is not permissible since the minimal rise in antibody titer considered diagnostically significant is a 4-fold rise, a change which is frequently encountered, but is detectable and valid only when every step of the complement fixation technic has been carefully performed

Procedures for titrating hemolysin and complement are so well known as to merit little comment here Aside from stressing the need for accuracy in all measurements, we would emphasize the desirability, if not the necessity, of always titrating complement in the presence of antigen

TABLE 6

Serologic Methods Applicable to Diagnosis of Specific Viral and Rickettsial Diseases

Disease	In Vitro Tests				In Vivo Tests (Neutralization Tests)	
	Type of Test					
	Complement Fixation	Hemagglutination Inhibition	Agglutination	Source of Antigen	Source of Virus	Test Species
Respiratory Group						
Influenza A	+	+		Allantoic fluid		
Influenza B	+	+		Allantoic fluid		
Influenza C	+	+		Membranes plus amniotic fluid		
Primary atypical pneumonia			Cold aggl Strep aggl	Human group O cells (hemagglutination) *Streptococcus* MG (agglutination)		
Adenovirus group	+			Tissue culture (He La cell) fluid	Tissue culture fluid	Tissue culture
Psittacosis-LGV group	+		+	Yolk sac		
Miscellaneous Group						
Colorado tick fever	+			Mouse brain	Mouse brain	Mouse
Coxsackie	+			Suckling mouse tissue	Suckling mouse tissue	Suckling mouse
Dengue	+			Mouse brain (suckling mice)	Mouse brain	Mouse
Herpes simplex	+			Membranes plus amniotic and allantoic fluids	Mouse brain Chorioallantoic membrane	Mouse Chorioallantois

Lymphocytic choriomeningitis	+			Mouse brain, guinea pig spleen	Mouse brain Guinea pig brain	Mouse Guinea pig
Mumps	+	+		Amniotic and allantoic fluid		
Newcastle disease	+	+		Allantoic fluid		
Poliomyelitis	+			Tissue culture fluids for Types I, II, and III, suckling mouse brain for Type II only	Tissue culture fluids Mouse brain	Tissue cultures Mice i c. or spinal
Vaccinia variola	+	+		Vesicles or crusts from patient (CF), membranes from eggs used for virus isolation (CF, HI)		
Yellow fever	+			Mouse brain	Mouse brain	Mouse
Encephalitis Group						
Eastern equine encephalomyelitis	+			Membranes plus amniotic and allantoic fluids, mouse brain	Mouse brain	Mouse
Japanese B encephalitis	+	+		Mouse brain	Mouse brain	Mouse
St. Louis encephalitis	+	+		Mouse brain	Mouse brain	Mouse
Venezuelan equine encephalomyelitis	+			Membranes plus amniotic and allantoic fluids, mouse brain	Mouse brain	Mouse
Western equine encephalomyelitis	+	+		Membranes plus amniotic and allantoic fluids, mouse brain	Mouse brain	Mouse
Rickettsial Group						
Q fever	+		+	Yolk sac		
Rickettsialpox	+			Yolk sac		
Rocky Mountain spotted fever	+		Weil Felix	Yolk sac, *B proteus* OX-19 and OX-2		
Typhus fever, epidemic and murine	+		+ and Weil Felix	Yolk sac, *B proteus* OX-19		

in the concentration in which the antigen is to be used in the test. Viral antigens have a tendency to combine with greater or lesser amounts of complement, and unless this is taken into account and compensated for, inadequate amounts of complement may be used in the test itself with all the attendant difficulties This is especially true where the fixation method employs two *exact* units of complement

The antigen represents one of the variables in the test, and its titration constitutes an important step. Two procedures are in common use, the so-called "straight-line" method and the "box" (or "checkerboard") titration. In the "straight-line" procedure, falling dilutions of antigen are tested against a single, arbitrarily chosen dilution of immune serum The unit is represented by the maximum dilution of antigen that will fix with the dilution of serum chosen In the test proper, the amount of antigen used is some such multiple of this unit as 2, 4, 6, et cetera. The number of units employed vary from method to method, there being no uniformity except that the upper limits are set by such properties of the antigen as its hemolytic, anticomplementary, or nonspecific activity. The "box" method of titration tests falling dilutions of antigen against falling dilutions of the immune serum, and since the unit represents the highest dilution of antigen that will give complete, or nearly complete, fixation with the highest dilution of serum, it thus represents what is essentially the optimal proportion between antigen and antibody.[25] The concentration of antigen represented by 1 unit therefore is adequate to give fixation with very low concentrations of antibody, whereas the straight-line method gives a unitage which may be inadequate to obtain the same result, hence the necessity of relatively high multiples of the "straight-line" unit in a test as compared to the usual 1½ to 2 units required on the basis of the "box" titration method

Sheep erythrocyte suspensions comprise a variable to which comparatively little attention has been paid, although it is an important one Preparation of the suspension requires care to prevent alterations in the cells that may affect the test Suspended cells can be made accurately to the desired concentration by packing to a minimal volume, a common procedure that, nevertheless, may produce either obvious destruction of cells as is indicated by visible hemolysis or, more importantly, may result in physicochemical changes which render the cells more susceptible to lysis A paper by Collier *et al*[25] describes a method by which maximal packing is not necessary In brief, the true hematocrit value of an aliquot of washed erythrocyte suspension is determined by

centrifuging it in a Bourke-Ernstene hematocrit tube, and from this value and that of the volume of the initial suspension, the dilution of the original suspension required to produce the desired concentration is computed. Suspensions prepared by this method are said to be reproducible both as to accuracy of concentration and as to susceptibility to lysis.

In regard to the test proper, a few points merit mention. Overnight incubation in the cold increases sensitivity as compared with brief incubation at 37° C. and is employed for this reason as well as for convenience Tests with a primary incubation period of 1 hour in the water bath, although less sensitive, can be used for diagnostic purposes provided their use conforms with the established principle that a diagnosis can be achieved only through the demonstration of a rise in antibody titer, a procedure which necessitates the use of serial blood specimens. The use of such tests for so-called "rapid diagnosis" or "emergencies" is based on the widespread but erroneous belief that an unequivocal diagnosis can be made on the basis of a "diagnostic titer" obtained through examination of a single specimen of serum and is to be discouraged. Because of the tendency of viral antigens, especially the cruder preparations, to fix complement to some extent, there should be included among the several usual controls a control on the amount of complement being bound by the antigen In general, a 4-tube complement control system is used, the tubes containing, before the addition of the hemolytic system, antigen, saline, and 2 0 units, 1 5 units, 1 0 unit, and 0 5 unit respectively of complement In systems employing *exact* units, complete hemolysis in the 3 tubes containing 1 0 or more units of complement indicates that the test system employed proper amounts of complement, any hemolysis in the tube containing 0.5 unit of complement indicates that an excess was used in the test and partial fixation in the tubes containing 1 0 or more units of complement indicates that inadequate amounts were used, the degree of insufficiency being indicated by the extent to which these tubes fail to show clearing

Nonspecific reactions, while more likely to occur when comparatively crude antigens are used, may be encountered at any time Consequently, each serum should have a control not only for anticomplementary activity but also for nonspecific reactivity The nonspecific control consists in testing the lowest dilution of serum used in the test proper against an antigen prepared from normal tissue processed by the same procedures as the test antigen or by using a heterologous (and anti-

genically *unrelated*) viral antigen prepared by the same method. Until relatively recently, the antigens used in many cases, primarily those prepared from mouse brain tissue, were rather crude and prone to give nonspecific reactions, in addition, they were also so weak antigenically that specific fixation could be obtained only with very low serum dilutions. The potency in some cases has been increased through the use of starting material with a high virus content, and specificity has been sharpened by purification of the antigens through such means as extraction with benzene or acetone or removal of nonspecific protein through precipitants or by high-speed centrifugation. The sources from which antigens are derived are illustrated in Table 6; details of preparation are given in the chapters dealing with specific diseases Application of the 50 per cent end point method to diagnostic work is an unnecessary refinement since a simple 4-fold rise in titer is sufficient on which to base a diagnosis Potent antigens, however, should be sought, since 4-fold rises may be of doubtful validity when the titers fall in the very low ranges, for example, a rise from 1·2 to 1.8. Because of the high proportion of nonspecific reactions that may occur with very low serum dilutions, we have adopted 1·8 as the initial serum dilution employed in the test

Anticomplementary reactions may be troublesome on occasion and can be divided into two groups according to the basis for the trouble[27] The so-called "intrinsic" group comprises those sera in which the anticomplementary activity is an inherent property of the serum of the individual and is not due to faulty technic In some patients, repeated bleedings will consistently yield sera which are anticomplementary, whereas in others the serum may be anticomplementary on some occasions, but not all A high proportion of persons yielding repeatedly anticomplementary sera has been found to have syphilis[27] The "extrinsic" group comprises those sera in which the anticomplementary action is caused by extraneous factors, namely, bacterial or chemical contamination Arsenicals, alcohol, anticoagulants (such as citrate, oxalate, and heparin), preservatives, and other substances may give rise to anticomplementary properties in the serum The predominant cause of anticomplementary activity is, in our experience, bacterial contamination of the specimen.

2. *Hemagglutination-inhibition tests* The basis for the hemagglutination test lies in the fact that certain viruses possess the capacity to agglutinate red blood cells and that specific antibody prevents or inhibits

hemagglutination, this reaction, therefore, can be utilized as a means of antibody assay After the discovery that influenza virus possesses hemagglutinative properties, investigations revealed that hemagglutination is a property of a large number of other viruses. The list now includes—in addition to the well-known examples of mumps, Newcastle disease, and agents of the vaccinia-variola group—the viruses of dengue, yellow fever, West Nile disease, and Japanese B, Russian Far East, Western equine, St Louis, and Murray Valley encephalitis

Since the actual mechanics of the hemagglutination-inhibition test are so simple, consisting merely of adding a standardized amount of virus to dilutions of the serum under test, incubating and subsequently adding erythrocytes of the appropriate animal species as a reaction indicator, it might seem, a priori, that so simple a test should supersede other methods However, even so widely used a test as the hemagglutination-inhibition method for influenza has its drawbacks, unless the technics described for the hemagglutination-inhibition test with some of the agents mentioned above can be shown to have decided advantages in detecting antibody sooner or in detecting significant rises in antibody earlier, and so on, their cumbersomeness or intricacy will favor use of the complement fixation test. If, however, future work shows that the hemagglutination-inhibiting antibody parallels neutralizing antibody in appearance and persistence, the inhibition test may supplant the neutralization test

Since the greatest amount of work on the hemagglutination phenomenon has been done with the influenza virus, this virus will serve to illustrate some of the difficulties associated with the hemagglutination-inhibition test One difficulty is that the test is relatively strain specific, a property which is utilized in detecting or demonstrating differences in antigenic constitution of strains of influenza virus From a diagnostic standpoint, such specificity is an undesirable characteristic, since, if the antigen used for antibody determination of a patient's serum is markedly different from contemporary strains involved in current outbreaks, detection of antibody rises may be missed In practical application, this means that the antigen used in the test must be broad and also that in each outbreak, or during each season, prevalent strains should be isolated and compared with the strains being currently used so that, if necessary, the inadequate test strains may be replaced. Usually, diagnostic hemagglutination-inhibition tests call for the use of several strains of both the Type A and Type B viruses, which is time-consuming, and the work load

is inordinately increased as compared to the complement fixation test. Since the complement fixation test is group specific, that is, it discloses *similarities* between *strains*, *it is simpler to do a single complement fixation* test utilizing a Type A and a Type B antigen. For typing of the outbreak, hemagglutination-inhibition tests may be done with randomly selected sera found positive by the complement fixation method.

Other difficulties lie in the existence of nonspecific inhibitors in human sera Again using influenza viruses as an example since the most detailed studies of inhibitors have been done with this agent, it may be pointed out that several inhibitors active against this virus are present in human sera. Some of these inhibitors are heat labile, and as they are destroyed by heating at 56° C for 30 minutes, they are of no consequence in the test (This provides another example of the advisability of inactivating sera before use in serologic tests.) At least 2 nonspecific inhibitors, however, which are heat stable are known and are of concern since they may profoundly affect the reliability of the test.[25] One of these, the alpha inhibitor, presumably identical with the so-called "Francis inhibitor," is rendered inert by a substance present in filtrates of *Vibrio cholerae*, designated as receptor-destroying enzyme (RDE). The other, or beta inhibitor, is not affected and is encountered to some extent in human sera, although the inhibitory action of such sera is due primarily to the alpha inhibitor.

The effect of nonspecific inhibitors in the test is important from several aspects. The sera of children may be high in nonspecific inhibitor content but essentially devoid of antibody for certain virus strains.[28] Also, inhibitors may prevent agglutination at much higher dilutions than does the specific antibody so that a true diagnostic rise in antibody titer may be masked and not detectable until the sera are treated with receptor-destroying enzyme Removal of nonspecific inhibitors from serum is thus important in determining the basic antibody levels in groups or populations for the various influenza strains It is obvious, therefore, that caution must be exercised in interpreting the results of hemagglutination-inhibition tests. Although the reliability and the sensitivity of the method for demonstrating specific antibody can be greatly increased *by pretreatment of sera with RDE, such manipulations add appreciably to the* time involved in conducting the tests. This objection may perhaps be overcome by the use of contemporary virus strains insensitive to the alpha inhibitor, such as the FW-1 (Cuppett) A-prime strain; no strains

insensitive to alpha inhibitor have as yet been found among the Type B viruses [28]

Problems associated with nonspecific inhibitors are by no means restricted to influenza. On the basis of data derived from the hemagglutination-inhibition technic, the viruses of mumps and Newcastle disease were considered to be antigenically related until recent work showed that this apparent relationship is spurious [29,30] It thus appears that a nonspecific inhibitor is present in many sera obtained from apparently healthy persons, and that in addition sera containing specific antibodies against mumps virus also contain a heat-stable, heterotypic inhibitor substance for Newcastle disease virus [29] The considerable frequency with which cross reactions between mumps and Newcastle disease viruses are observed in hemagglutination-inhibition tests must be kept in mind, the problem of cross reactions can be largely bypassed through use of the complement fixation in which cross reactions are only occasionally encountered.[30]

The hemagglutination-inhibition tests for such diseases as dengue, yellow fever, and certain neurotropic virus infections are still so new that unqualified statements regarding their value and usefulness in diagnostic work cannot be made until current information is considerably amplified by more extensive experience. The various methods as they stand at the present time, however, are unsatisfactory for applied diagnostic work because of their cumbersomeness or intricacy, or both Thus, in the St Louis virus hemagglutination-inhibition test, rapidity of manipulation is required to avoid loss of the hemagglutinin, which is highly unstable, and rigid and exacting control of the pH range and other conditions under which various steps of the test are conducted must be maintained

In the group of hemagglutination-inhibition tests under consideration here, the hemagglutinin is derived from mouse brain infected with virus In addition to the presence of *specific hemagglutinin*, such infected brains contain, just as do normal mouse brains, a *nonspecific hemagglutinin* for erythrocytes from a variety of animal species [31,32] Moreover, there may be present concomitantly with these nonspecific hemagglutinins, a thermolabile *inhibitor* of agglutination [33,34] It becomes necessary, as a consequence, to control thoroughly the variables in each test in order to avoid misinterpretations arising from nonspecific, nonviral hemagglutination. The need for care, accuracy, and precision is exempli-

fied by the technics described for the dengue and St. Louis encephalitis hemagglutination-inhibition tests.

Unlike the nonspecific inhibitors of influenza virus, which are mucoprotein, the inhibitors associated with viruses propagated in mouse brain apparently are lipoidal. Recent work indicates [35] that with some viruses the nonspecific inhibitors can be removed by extraction of the mouse brain tissue with acetone and ether. Because of the high hemagglutinin content of such extracted tissue, the use of these relatively inhibitor-free antigens would appear feasible. However, a practical difficulty lies in the fact that thermostable inhibitors are present in serum, both human and animal.[33,34] The inhibitor, which is a lipid or lipo-protein, can be removed from serum by repeated extraction with acetone [34,35] Extraction of each serum to be tested is hardly a practicable procedure in large-scale, applied diagnostic work, and unless hemagglutination-inhibition tests utilizing mouse brain antigens can be shown to have decided immunologic advantages over the simpler complement fixation technic, they are not likely to completely replace them at the present time.

3 *Agglutination tests.* Agglutination tests may be divided into two categories as to whether they are "biologically specific" or "biologically nonspecific."

While biologically specific agglutination tests are feasible with the larger viruses, such as those of psittacosis and vaccinia, and with the rickettsiae, they are but little employed in diagnostic work. Comparatively pure suspensions of some of these agents are difficult to prepare, but even where this is feasible, rather large and hence expensive amounts of antigen are utilized Microscopic agglutination tests of one type or another have been described, but in general find little favor because of the large error associated with quantitative micromethods. Agglutination tests do, however, have a place as, for example, under operating conditions where complement fixation methods are not feasible but, even so, macroscopic methods are preferred. Also, in the rickettsioses, agglutination tests provide an alternate procedure when the serum to be examined is anticomplementary.

Of the biologically nonspecific agglutination tests, the Weil-Felix method for the diagnosis of rickettsial diseases of the spotted fever and typhus groups is probably the best known and is still widely used. Following the development of specific complement fixation methods for the rickettsioses, this procedure in many laboratories replaced the Weil-Felix method, entirely or in part. Care and caution were, and are, re-

quired in interpreting the findings of the Weil-Felix test since a positive finding may be due not only to faulty technic or improperly prepared antigen, but also is not infrequently encountered in conditions other than rickettsial infections, for example, *Proteus* infections, prostatitis, and pregnancy.

It has been reported recently that the complement fixation test may fail to demonstrate the appearance or rise in titer of antibodies in what clinically is undoubtedly a rickettsial disease, whereas the Weil-Felix reaction is positive. The discrepancy in the results of the two tests appears attributable to interference with antibody formation in those patients who receive early and intensive antibiotic therapy; complete suppression, or almost complete suppression, of complement-fixing antibody formation may occur, whereas the formation of the X-agglutinins on which the Weil-Felix reaction is based is little, if at all, affected. Just as the findings of the Weil-Felix test should be interpreted in the light of the patient's clinical and epidemiologic background to rule out false positives, so should the negative results of the complement fixation test be checked by the Weil-Felix method when clinical and epidemiologic findings point strongly to the existence of a rickettsial infection.

The cold agglutination test and the Streptococcus MG agglutination test are biologically nonspecific methods used in the diagnosis of primary atypical pneumonia. Agglutinins to group O human erythrocytes or to the Streptococcus MG appear in the blood of many patients during the course of this disease. Cold agglutinins, as their name implies, possess the capacity to agglutinate erythrocytes in the cold (icebox temperature); hence, serum to be used for this test should be removed from clotted blood which has not been refrigerated or, if it has been refrigerated, it should be allowed to come to room temperature before removal of the serum in order to permit elution of adsorbed agglutinins. With the Streptococcus MG agglutination test, stock cultures must be properly maintained and test suspensions appropriately prepared to avoid spontaneous agglutination. On the whole, approximately only 50 per cent[15] of the patients develop agglutinins for either group O human erythrocytes or the Streptococcus MG, and hence a positive test constitutes supportive evidence for the clinical diagnosis, whereas a negative test does not rule out the disease. In addition, it has also been observed that antibiotic therapy may interfere with the development of cold agglutinins.[16]

It should be remembered that the cold agglutination test is positive

in a number of diseases other than primary atypical pneumonia and that this test, as well as the Streptococcus MG test, has recently been found to give positive results in gastroenteritis of children.[37]

4 *Neutralization tests.* The neutralization test is based on the principle that when a specific immune serum is added to its corresponding virus, the virus is rendered noninfective or "neutralized" Since demonstration of infectivity requires the use of living tissues, the host systems employed are embryonated eggs, various animal species, and tissue cultures.

In ovo neutralization tests have been used primarily in investigative work, they are used to only a limited extent in diagnostic laboratories

Three types of end point are employed in *in ovo* neutralization tests to determine the neutralizing capacity of sera The first of these end points rests on the ability of the virus to produce plaques or pocklike lesions on the chorioallantoic membrane. The degree of neutralization which occurs is based on the quantitative reduction in number of virus particles capable of producing lesions, which is indicated by the number of plaques produced The procedure is time-consuming and open to a number of errors,[38] and reading and interpretation of the findings require considerable experience Determination of the infective titer of the virus alone is apt to yield variable results, estimation of infectivity in the presence of serum gives rise to an additional error arising from the lack of proportionality between virus dilutions and plaque counts[39]

A more accurate end point for the quantitative measurement of antibody is that based on the lethal action of serum-virus mixtures. However, infections with virtually all the viruses which produce lethal effect in the chick embryo are also amenable to diagnosis by in vitro serologic methods, which are simpler

The third end point, feasible only with those viruses which produce hemagglutinins, depends upon the appearance or nonappearance of hemagglutinins in individual eggs inoculated with the serum-virus mixture The need for examining each egg, or group of eggs, for the presence of hemagglutinins adds considerably to the work load, and the same diagnostic information can be obtained more readily and quickly by in vitro tests

While animals other than the albino Swiss mouse are used for neutralization tests, their use is reserved for certain situations and circumstances in which the mouse cannot be employed. Since most of the commonly encountered viruses are pathogenic for the Swiss mouse, this species is employed in diagnostic work almost to the exclusion of all others Selectively inbred strains of a high and uniform susceptibility should be used.

The age of the test animal is of importance under some conditions Both suckling and adult mice are about equally susceptible to cerebral infection with

many neurotropic agents, the very immature animals being on the whole perhaps slightly more susceptible. There are some agents, however, such as the dengue virus and the Coxsackie viruses, which are pathogenic by the cerebral route only for the very immature mouse and not for the mature animal; consequently, neutralization tests with these agents require suckling animals of a susceptible age Other agents, pathogenic for both adult and immature mice on cerebral inoculation, are lethal by the peripheral route only for the very young animal This characteristic is utilized in the extraneural neutralization test, which in many cases is a far more sensitive method for detection and quantitation of antibody than is the usual intracerebral method [40,41] There are also indications that age may play a role in the susceptibility of the chick embryo to infection by different routes of inoculation [42]

There exists a wide divergence in the methodology associated with the conduct of neutralization tests Certain variations might be expected, such as differences in hosts because of their differences in susceptibility and differences in the route of inoculation because of differences in the pathogenic properties between agents On the other hand, there exist such differences between tests as the ratio of virus to serum volumes employed, whether or not incubation is used, if incubation is used, there are differences in the time and the temperature employed; and between the use of varying virus-constant serum or constant virus-varying serum dilutions Some of these differences are based on empirical observations, others have been the subject of limited investigation Because of the differences of opinion and in findings recorded in the literature and lack of information as to the need or validity for some of the procedures, it is impossible to recommend one or another method as being superior to all others and feasible in all instances The only advice which can be given at the present time is to follow those technics recommended for each specific viral infection.

Whether incubation of serum-virus mixtures is necessary is a matter on which investigators differ Some workers report finding no difference in neutralizing capacity of a serum, whether or not incubation is used, others believe that incubation produces only varying degrees of secondary inactivation of the virus, and still others maintain that incubation enhances the neutralizing effect In some instances, it would appear that incubation is mandatory, or virtually so, in order to reveal a neutralization effect (see, for example, Sabin [43])

Under the section on the collection and handling of blood specimens, there was mentioned the widely accepted practice that sera for neutralization tests (and for other serologic tests, for that matter) should be removed from the clot promptly and stored in the frozen state This procedure is based on observations that the neutralizing capacity falls off if the serum is stored at the usual icebox temperature of 4° to 6° C., the end result being that the neutralizing capacity of a stored acute phase serum may be lower than that of a relatively fresh convalescent phase serum

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The age of the test animal is of importance under some conditions. Both suckling and adult mice are about equally susceptible to cerebral infection with

phase serum specimens may have high and essentially the same antibody titers, that is, diagnostically significant differences in antibody titer between the two specimens are not demonstrable. The constant virus-varying serum method, on the other hand, appears to be superior in several respects[45] Measurement of antibody levels is more accurate because even large variations in amount of virus used produce only small changes in the titer of the serum[45] Moreover, a wide range of serum dilutions can be examined, and the method permits the use of viruses with low titers. The constant virus-varying serum method probably will find increasingly greater acceptance.

VI. COMPUTATION OF 50 PER CENT END POINTS

A. General Comments

In biological quantitation, the end point is usually taken as the dilution at which a certain proportion of the test animals reacts or dies While a 100 per cent end point still is not infrequently used, its accuracy is so grossly affected by small chance variations as to make it the worst type of end point. The most desirable is one representing a situation in which one-half of the animals react, the other one-half do not[47] The best method of determining such an end point is to use large numbers of test animals at closely spaced dilutions near the value for 50 per cent reaction, and then interpolate a correct value A number of practical factors, however, militate against such an approach: (a) the cost of using large numbers of animals on every dilution point; (b) the wide variations in titer between any given tests, and (c) in most instances, the unjustified application of highly accurate statistical methods to procedures replete with uncontrolled variables.

In titrating viruses or, for example, sera for antibody content, a series of dilutions of the test materials is made, and each dilution is inoculated into a small group of animals, ordinarily 6 to 8 animals are used on each "point," *in toto*, therefore, employing a large number of animals. Reed and Muench[48] have devised a simple method for estimating 50 per cent end points based on the large, *total* number of animals, which gives the effect "of using, at the 2 critical dilutions between which the endpoint lies, larger groups of animals than were actually included at these dilutions. By inclining to equalize chance variations, the method tends to define the point more nearly than would be possible if it were

and may thus indicate that a rise in antibody titer apparently occurred in the interval between the two bleedings The work of Ginsberg and Horsfall[7] points to the existence in such sera of a nonspecific viral inhibitor since they found that normal human and animal sera possess the capacity in variable degree to neutralize the viruses of influenza, Newcastle disease, and mumps and that this capacity disappears when the sera are heated (56° C. for 30 minutes) These authors state that it is hazardous to use unheated sera in neutralization tests Still, in some circumstances it may appear desirable or even necessary to use only frozen sera or to add fresh, unheated sera to test sera which have been inactivated For example, neutralization of the mouse-adapted dengue virus by intracerebral tests in mice has been found by Sabin[43] to depend on two factors, namely, a specific antibody which is heat stable (56° C. for 30 minutes) and a nonspecific, heat-labile accessory substance. The neutralizing capacity of heated dengue- immune serum is fully restored by fresh animal serum, which in itself has no neutralizing activity This accessory factor thus has a potentiating and not an inhibitory action. A similar potentiating effect has been described for vaccinia virus by McCarthy and Germer[8] (According to Sabin,[43] not only must the accessory factor be present but incubation of the serum-virus mixtures is mandatory) Smithburn[44] also states that heating seriously diminishes the neutralizing capacity of St Louis immune sera and that the addition of fresh serum to such inactivated test sera will restore much of the original neutralizing activity.

Obviously, additional research is desirable to determine definitively whether or not incubation is necessary for any one particular neutralization test, and on the subject of nonspecific inhibitors and accessory or potentiating factors in sera Using fresh, unheated sera in neutralization tests may give misleading results if nonspecific inhibitors are present. Similarly, if test sera are heated to destroy nonspecific inhibitors, and unheated normal serum is added to enhance the neutralizing activity, one must be sure that the added serum contains a potentiating factor and not a nonspecific inhibitor.

There is, finally, one very wide difference between neutralization tests, which is so fundamental that further studies on the subject are most desirable. This difference lies in whether undiluted serum is tested against serial dilutions of virus or whether dilutions of serum are tested against a constant dose of virus. Since space does not permit detailed discussion here, the reader is referred to the recent publications of Tyrrell and Horsfall[45] and Tyrrell[46] on this subject. The most commonly employed technics use the constant serum-varying virus method. By this procedure, a large change in virus titer is required to reveal small differences in antibody concentration, and this probably explains the many instances in which it is difficult or impossible to make a diagnosis by this neutralization method, since both the acute and the recovery

TABLE 7

ARRANGEMENT OF DATA USED IN COMPUTATION OF LD_{50} TITER BY REED-MUENCH FORMULA

Virus Dilution a	Mortality Ratio b	Died c	Survived d	Accumulated Values: Died e	Accumulated Values: Survived f	Mortality: Ratio g	Mortality: Per Cent h
10^{-1}	6/6	6 ↑	0	17	0	17/17	100
10^{-2}	6/6	6	0	11	0	11/11	100
10^{-3}	4/6	4	2	5	2	5/7	71
10^{-4}	1/6	1	5	1	7	1/8	13
10^{-5}	0/6	0	6 ↓	0	13	0/13	0

50 per cent, that in the next lower dilution, 10^{-4}, is considerably below The necessary proportionate distance of the 50 per cent mortality end point, which obviously lies between these two dilutions, is obtained as follows

$$\frac{\text{(Per cent mortality at dilution next above 50\%)} - (50\%)}{\text{(Per cent mortality at dilution next above 50\%)} - \text{(per cent mortality at dilution next below)}} = \text{Proportionate distance}$$

$$\text{or} \quad \frac{71-50}{71-13} = \frac{21}{58} = 0.36 \text{ (or 0.4)}$$

Since logarithmically the distance between any 2 dilutions is a function of the incremental steps used in preparing the series, for example, 2-fold, 4-fold, 5-fold, 10-fold, etc , it is necessary to correct (multiply) the proportionate distance by the dilution factor, which is the logarithm of the dilution steps employed In the case of serial 10-fold dilutions, the factor is 1 ($\log 10 = 1$) and so is disregarded, in a 2-fold dilution series, the factor is 0.3 (log of 2.0), in a 5-fold series, 0.7 (log of 5.0), and so on In the procedure which follows, the factor is understood to be negative Therefore,

Negative logarithm of LD_{50} end point titer = negative logarithm of the dilution above the 50 per cent mortality plus the proportionate distance factor (corrected for dilution series used)

or

Negative logarithm of the lower dilution (next above 50% mortality)	= −3.0
Proportionate distance (0.4) × dilution factor (log 10)	= −0.4
log LD_{50} titer	= −3.4
LD_{50} titer	= $10^{-3.4}$

simply interpolated between the 2 bracketing results."[48] Kärber[49] also has reported a simple method for computing 50 per cent end points; this will be described below, along with the Reed-Muench method.*

The 50 per cent end point can be based on several types of reactions. The most widely used is based on *mortality* and is written LD_{50} (50 per cent lethal dose) ID_{50} indicates the dose which *infects* 50 per cent of the test animals, PD_{50} the dose which *paralyzes* 50 per cent of the animals, et cetera. The terminology can also be applied to other host systems, for example, tissue cultures, in which TCD_{50} represents the dose that gives rise to cytopathic changes in 50 per cent of the inoculated cultures.

Both the Reed-Muench and the Karber methods are applicable primarily to a complete titration series, that is, the whole reaction range, from 0 per cent to 100 per cent mortality (or infectivity or cytopathic effect, etc), should be represented in the experimental data. However, the methods can be utilized even if these conditions are not fulfilled, provided the reactions occur in a uniform manner over the range of dilutions employed. If, however, these are erratic (for example, deaths irregularly scattered over a number of dilutions), the end point will be inaccurate There are, of course, a number of other statistical methods which can be employed (Bross[50]), but they are not so commonly used in the virus literature.

B Fifty Per Cent End Points in Virus Titrations

1. *Calculation of the LD_{50} titer by the Reed-Muench method.* If the laboratory work sheets or cards are provided with an appropriate column at one side, the accumulated values (Table 7, columns e-h) can be quickly calculated and the end point determined as outlined below. Table 7 gives an example of data derived from observation of inoculated animals and illustrates the procedure of accumulation.

Accumulated values for the total number of animals that died or survived are obtained by adding in the directions indicated by the arrows The accumulated mortality ratio (column g) represents the accumulated number of dead animals (column e) over the accumulated total number inoculated (column e plus column f); for example, in the 10^{-3} dilution, there were 5 deaths out of a total of 7 animals.

In the example in Table 7, the mortality in the 10^{-3} dilution is above

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* We are indebted to Dr Joseph Melnick and Dr John Paul for bringing the Karber method to our attention for inclusion here.

TABLE 7

ARRANGEMENT OF DATA USED IN COMPUTATION OF LD_{50} TITER BY REED-MUENCH FORMULA

Virus Dilution a	Mortality Ratio b	Died c	Survived d	Accumulated Values: Died e	Accumulated Values: Survived f	Accumulated Values: Mortality Ratio g	Accumulated Values: Mortality Per Cent h
10^{-1}	6/6	6	0	17	0	17/17	100
10^{-2}	6/6	6	0	11	0	11/11	100
10^{-3}	4/6	4	2	5	2	5/7	71
10^{-4}	1/6	1	5	1	7	1/8	13
10^{-5}	0/6	0	6	0	13	0/13	0

50 per cent, that in the next lower dilution, 10^{-4}, is considerably below. The necessary proportionate distance of the 50 per cent mortality end point, which obviously lies between these two dilutions, is obtained as follows

$$\frac{(\text{Per cent mortality at dilution next above } 50\%) - (50\%)}{(\text{Per cent mortality at dilution next above } 50\%) - (\text{per cent mortality at dilution next below})} = \text{Proportionate distance}$$

or $$\frac{71-50}{71-13} = \frac{21}{58} = 0.36 \text{ (or 0.4)}$$

Since logarithmically the distance between any 2 dilutions is a function of the incremental steps used in preparing the series, for example, 2-fold, 4-fold, 5-fold, 10-fold, etc., it is necessary to correct (multiply) the proportionate distance by the dilution factor, which is the logarithm of the dilution steps employed. In the case of serial 10-fold dilutions, the factor is 1 (log 10 = 1) and so is disregarded, in a 2-fold dilution series, the factor is 0.3 (log of 2.0), in a 5-fold series, 0.7 (log of 5.0), and so on. In the procedure which follows, the factor is understood to be negative. Therefore,

Negative logarithm of LD_{50} end point titer = negative logarithm of the dilution above the 50 per cent mortality plus the proportionate distance factor (corrected for dilution series used)

or

Negative logarithm of the lower dilution (next above 50% mortality)	= −3.0
Proportionate distance (0.4) × dilution factor (log 10)	= −0.4
log LD_{50} titer	= −3.4
LD_{50} titer	= $10^{-3.4}$

2. *Calculation of the LD_{50} titer by the Kärber method.* This method gives end points which are essentially as accurate as those obtained by the Reed and Muench method. It is not necessary to use accumulated mortality ratios (although these can be used), as the observed mortality ratios suffice.

The Kärber formula is as follows:

$$\text{Negative logarithm of the } LD_{50} \text{ end point titer} = \left[\begin{matrix}\text{negative logarithm of the highest}\\ \text{virus concentration used}\end{matrix}\right] - \left[\left(\frac{\text{sum of per cent mortality at each dilution}}{100} - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Using the data in Table 7, this becomes:

$$\begin{aligned}\text{Negative logarithm of the } LD_{50} \text{ end point titer} &= -1.0 - \left[\left(\frac{100+100+66+17}{100} - 0.5\right) \times (\log 10)\right]\\ &= -1.0 - \{(2.8 - 0.5) \times 1\}\\ &= -1.0 - 2.3\\ &= -3.3\\ LD_{50} \text{ titer} &= 10^{-3.3}\end{aligned}$$

C. Fifty Per Cent End Points in Neutralization Tests

1. *Determination of antibody end point in systems using varying doses of virus and constant amounts of serum (varying virus-constant serum method)*

This is the most commonly employed method and consists of adding a constant amount of the serum under test to decreasing amounts of virus. Generally, serum is added to virus in equal proportions, and the *mixtures, with or without incubation, are then inoculated into the test* host. The results are expressed in the form of a neutralization index, which indicates the capacity of the serum to neutralize the action of the virus on the host system employed. The neutralization index represents the difference (without regard to sign) in the titer of the virus in the presence of normal control serum and in the presence of the test (immune) serum. It is obtained by subtracting the logarithm of the LD_{50} titer of the immune (or test) serum from the logarithm of the LD_{50} titer of normal control serum. The index can be used either as a logarithm expressing the number of LD_{50} neutralized by the particular serum, or can be converted to the antilogarithm if it is desired to express the degree of neutralization in arithmetic terms.

Calculation of the neutralization index by the Reed-Muench method is illustrated by the data in Table 8.

TABLE 8

COMPUTATION OF NEUTRALIZATION INDEX BY REED-MUENCH FORMULA

Dilution Virus in Virus-Serum Mixture	Mortality Ratio	Died	Survived	Accumulated Values: Died	Accumulated Values: Survived	Mortality: Ratio	Mortality: Per Cent
			Normal Serum				
10^{-3}	6/6	6	0	14	0	14/14	100
10^{-4}	6/6	6	0	8	0	8/8	100
10^{-5}	2/6	2	4	2	4	2/6	33
10^{-6}	0/6	0	6	0	10	0/10	0
			Immune Serum				
10^{-1}	6/6	6	0	11	0	11/11	100
10^{-2}	5/6	5	1	5	1	5/6	83
10^{-3}	0/6	0	6	0	7	0/7	0
10^{-4}	0/6	0	6	0	13	0/13	0
10^{-5}	0/6	0	6	0	19	0/19	0

a Reed-Muench method

Normal control serum

Proportionate distance $= \frac{100-50}{100-33} = \frac{50}{67} = 0.8$

Negative logarithm of LD_{50} titer = negative logarithm of dilution above 50 per cent mortality + (proportionate distance × log dilution factor)

$= -4.0 + (0.8 \times \log 10)$

$= -4.0 + (0.8 \times -1)$

$= -4.0 + (-0.8)$

LD_{50} titer $= 10^{-4.8}$

Immune serum

Proportionate distance $= \frac{83-50}{83-0} = \frac{33}{83} = 0.4$

Negative logarithm of LD_{50} titer $= -2.0 + (0.4 \times \log 10)$

$= -2.0 + (0.4 \times -1)$

$= -2.0 + (-0.4)$

LD_{50} titer $= 10^{-2.4}$

Neutralization index $= 4.8 - 2.4$

$= 2.4$ logarithms of virus neutralized

$= 250$ LD_{50} of virus neutralized

b. Kärber method

Normal control serum

Negative logarithm of LD_{50} titer $= -3 - \left[\left(\frac{100+100+33}{100} - 0.5 \right) \times (\log 10) \right]$

$= -3 - [(2.33 - 0.5) \times 1]$

$= -3 - 1.8$

$= -4.8$

LD_{50} titer $= 10^{-4.8}$

Immune serum

Negative logarithm of LD_{50} titer $= -1 - \left[\left(\frac{100+83}{100} - 0.5 \right) \times (\log 10) \right]$

$= -1 - [(1.8 - 0.5) \times 1]$

$= -1 - 1.3$

$= -2.3$

LD_{50} titer $= 10^{-2.3}$

Neutralization index $= 4.8 - 2.3$

$=$ 2.5 logarithms of virus neutralized

$=$ 320 LD_{50} of virus neutralized

2 *Determination of antibody end point in systems using constant doses of virus and varying dilutions of serum (constant virus-varying serum method)*

A suitable test dose of virus is selected, for example, 300 LD_{50}, and falling dilutions of serum are added in equal amounts to the appropriate virus dilution The mortality in the inoculated animals is recorded, and the neutralizing end point is expressed as that dilution of serum which "protects" 50 per cent of the animals against the test dose of virus used.

TABLE 9

CALCULATION OF 50 PER CENT SERUM NEUTRALIZATION END POINT BY REED-MUENCH FORMULA

Dilution of Serum (Virus Dose Constant)	Mortality Ratio	Died	Survived	Accumulated Values: Died	Accumulated Values: Survived	Accumulated Values: Mortality Ratio	Accumulated Values: Mortality Per Cent
1:4 ($10^{-0.6}$)	0/6	0	6	0	17	0/17	0
1:16 ($10^{-1.2}$)	0/6	0	6	0	11	0/11	0
1:64 ($10^{-1.8}$)	2/6	2	4	2	5	2/7	29
1:256 ($10^{-2.4}$)	5/6	5	1	7	1	7/8	88
1:1024 ($10^{-3.0}$)	6/6	6	0	13	0	13/13	100

a. Reed-Muench method

From Table 9,

Proportionate distance =

$$\frac{50\% - (\text{mortality at dilution next below})}{(\text{mortality next above}) - (\text{mortality next below})} = \frac{50-29}{88-29} = \frac{21}{59} = 0.4$$

Logarithm 50 per cent neutralizing end point = $-1.8 +$ (proportionate distance × logarithm dilution factor)

$= -1.8 + [0.4 \times (-0.6)]$

$= -1.8 + (-.2)$

$= -2.0$

50 per cent neutralizing end point = antilogarithm of $-2.0 = .01$

Dilution = 1:100

b. Karber method

$$\text{Logarithm 50 per cent neutralizing end point} = -3.0 - \left[(-0.6) \left(\frac{33+83+100}{100} - 0.5 \right) \right]$$

$$= -3.0 - [(-0.6)(2.2 - 0.5)]$$

$$= -3.0 - [(-0.6)(1.7)]$$

$$= -3.0 + 1.0$$

$$= -2.0$$

50 per cent neutralizing end point = antilogarithm of $2.0 = .01$

Dilution = 1:100

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VI. REFERENCES

1 Lennette, E H An Evaluation of Diagnostic Procedures for Viral and Rickettsial Diseases, Chapter 26 in the *International Symposium The Dynamics of Virus and Rickettsial Infections*, ed. by F W Hartman, F L. Horsfall, Jr., and J G [illegible]

2 [illegible]

3 [illegible] *[illegible] and Med* [illegible] 54–85, 1945

4 Rogers, N G The Effect of Merthiolate on the Infectivity of Certain Viruses. *J Lab and Clin Med*, 35 433–435, 1951

5 Morgan, I M Quantitative Study of the Neutralization of Western Equine Encephalomyelitis Virus by Its Antiserum and the Effect of Complement *J Immunol*, 50 359–371, 1945

6 Whitman, L The Neutralization of Western Equine Encephalomyelitis Virus by Human Convalescent Serum The Influence of Heat Labile Substances in Serum on the Neutralization Index. *J Immunol.*, 56 97–105, 1947

7. Ginsberg, H. S, and Horsfall, F. L, Jr A Labile Component of Normal Serum [illegible]

8. [illegible]

9. [illegible]

10. Allen, E G, Kaneda, B, Girardi, A J, Scott, T. F. McN., and Sigel, M. M Preservation of Viruses of the Psittacosis-Lymphogranuloma Venereum Group and Herpes Simplex Under Various Conditions of Storage *J Bact*, 63 369–376, 1952.
11. Horsfall, F L., Jr. A Low Temperature Storage Cabinet for the Preservation of Viruses. *J. Bact*, 40 559–568, 1940.
12. Horsfall, F L, Jr, and Ginsberg, H S An Improved CO_2 Cabinet for Low Temperature Storage of Infectious Agents with Gaseous CO_2 Excluded from the Specimen Compartment *J Bact*, 61 443–451, 1951
13. Van Rooyen, C E, and Rhodes, A. J *Virus Diseases of Man* 2d ed New York, Thomas Nelson and Sons, 1948.
14. [illegible] *Virology* 2d ed. Baltimore, The [illegible]
15. [illegible] Virus Infections *Bull New York* [illegible]
16. [illegible] Age on the Susceptibility of Mice to Infection with Certain Neurotropic Viruses *J Immunol*, 49 175–191, 1944
17. Le [illegible]
18. Co [illegible]
19. Cottral, G E Endogenous Viruses in the Egg *Ann New York Acad Sc*, 55 221–235, 1952
20. Enders, J F, Weller, T H, and Robbins, F. C Cultivation of the Lansing Strain [illegible]
21. [illegible]
22. [illegible]
23. [illegible]
24. [illegible] Methods for Its Detection, in *World Health Organization Monograph Series*, No 19, 1953, pp 179–191
25. Collier, F. C, Reich, A, and King, J W The Importance of the Sheep Cell Suspension in the Complement Fixation Reaction Description of an Accurate Technique for the Preparation of a Uniform Cellular Suspension *J Lab and Clin Med*, 39 778–784, 1952
26. Dean, H R, and Webb, R A The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction *J Path and Bact*, 29 473–492, 1926.
27. Lighter, A G Observations on Anticomplementary Reactions *Arch Dermat. and Syph*, 67 362–368, 1953
28. Hilleman, M R, and Werner, J H Influence of Non-Specific Inhibitor on the [illegible] a *J Immunol*, 71:110–117, 1953.
29. [illegible] A Study of Infections Caused by [illegible] ific and Non-Specific Serologic Rela- [illegible]

30 Wenner, H A, Monley, A, and Jenson, M H A Study of Infections Caused by Mumps and Newcastle Disease Viruses II Some Properties of Specific and Non-Specific Serum Hemagglutination Inhibitor Components *J. Immunol*, 68 357–368, 1952.

31. Verlinde, J D, and de Baan, P Sur l'hémagglutination par des virus poliomyélitiques murins et la destruction enzymatique des récepteurs de virus poliomyélitique de la cellule réceptive *Ann Inst Pasteur*, 77 632–641, 1949

32 Yager, R H, Kuttler, K L, Olitsky, P K, and Murphy L C Hemagglutination by Neurotropic Viruses *U S Armed Forces M J*, 1 920–925, 1950

33 Olitsky, P K, and Yager, R H Hemagglutination by Columbia SK Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses Experiments with Other Viruses *Proc Soc Exper Biol and Med*, 71 719–724, 1949

34

35

36 Gallagher, C D, and Gallagher, J R Effect of Aureomycin on the Development of Cold Agglutinins in Primary Atypical Pneumonia. *New England J Med*, 247 314–316, 1952

37 Keitel, H G Occurrence of Cold and Streptococcus MG Agglutinins in Infants with Gastroenteritis *J Inf Dis* 86 219–222, 1950

38 Burnet, F M, and Faris, D D The Technique of Quantitative Chorio-Allantoic Virus Titration *J Bact* 44 241–248 1942

39 Jawetz, E, and Coleman, V R Studies on Herpes Simplex Virus III The Neutralization of Egg-Adapted Herpes Virus by Human Sera *in Ovo* *J Immunol*, 68 645–653, 1952

40 Lennette E H, and Koprowski, H Neutralization Tests with Certain Neurotropic Viruses A Comparison of the Sensitivity of the Extraneural and Intracerebral Routes of Inoculation for the Detection of Antibodies *J Immunol* 49 375–385, 1944

41 Lennette E H and Koprowski H Comparative Sensitivity of the Extraneural and Intracerebral Neutralization Tests in Following the Antibody Response in Man to Vaccination with Western Equine Encephalomyelitis Virus *J Immunol*, 52 343–353, 1946

42 Crawley J F Neutralization Tests with Eastern Equine Encephalomyelitis Virus in the Chick Embryo A Statistical Treatment of the Results by the Probit Method *J Immunol* 59 319–330 1948

43 Sabin, A B The Dengue Group of Viruses and Its Family Relationships *Bact Rev*, 14 225–232 1950

44 Smithburn K Personal communication 1953

45 Tyrrell, D A J and Horsfall F L Jr Neutralization of Viruses by Homologous Immune Serum I Quantitative Studies on Factors Which Affect the Neutralization Reaction with Newcastle Disease Influenza A, and Bacterial Virus T *J Exper Med*, 97 845–861 1953

46 Tyrrell, D A J Neutralization of Viruses by Homologous Immune Serum II Theoretical Study of the Equilibrium State *J Exper Med.*, 97 863–870, 1953

47 Gaddum J H Reports of Biological Standards III Methods of Biological Assay Depending on a Quantal Response Med. Res Council, London *Special Report Series* No 183 1933

48 Reed L J and Muench H A Simple Method of Estimating Fifty Per Cent End points *Am J Hyg* 27 493–497 1938

49 Kärber G Beitrag zur kollecktiven Behandlung pharmakologischer Reihenversuche *Arch f exper Path u Pharmakol* 162 480–483 1931.

50 Bross I Estimates of the LD_{50} A Critique *Biometrics* 6 413–423, 1950

7. Ginsberg, H S, and Horsfall, F. L, Jr A Labile Component of Normal Serum Which Combines with Various Viruses. Neutralization of Infectivity and Inhibition of Hemagglutination by the Component. *J. Exper. Med*, 90.475–495, 1949
8. McCarthy, K, and Germer, W. D. Two Heat-Labile Factors in Normal Sera Which Neutralize Variola Virus. *Brit J. Exper. Path*, 33 529–536, 1952.
9. Ohtsky, P. K, Casals, J., Walker, D. L, Ginsberg, H S., and Horsfall, F. L, Jr. Preservation of Viruses in a Mechanical Refrigerator at −25° C. *J. Lab. and Clin Med*, 34 1023–1026, 1949
10. Allen, E G, Kaneda, B, Girardi, A J, Scott, T F. McN, and Sigel, M. M ... Group and ...–376, 1952
11. ... servation of
12. Horsfall, F L, Jr, and Ginsberg, H S An Improved CO_2 Cabinet for Low Temperature Storage of Infectious Agents with Gaseous CO_2 Excluded from the Specimen Compartment *J Bact*, 61 443–451, 1951.
13. Van Rooyen, C E, and Rhodes, A J *Virus Diseases of Man* 2d ed. New York, Thomas Nelson and Sons, 1948
14. ... *Virology* 2d ed. Baltimore, The
15. ... Virus Infections *Bull. New York*
16. Lennette, E H, and Koprowski, H Influence of Age on the Susceptibility of Mice to Infection with Certain Neurotropic Viruses *J Immunol*, 49.175–191, 1944
17. Lennette, E. H Isolation of St Louis Encephalitis Virus from a Fatal Human Case in California *Proc Soc Exper Biol and Med*, 61 206–210, 1946
18. Cox, H R Growth of Viruses and Rickettsiae in the Developing Chick Embryo *Ann New York Acad Sc*, 55 236–247, 1952.
19. Cottral, G E Endogenous Viruses in the Egg *Ann New York Acad Sc.*, 55·221–235, 1952.
20. Enders, J F, Weller, T H, and Robbins, F C Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues *Science*, 109 85–87, 1949.
21. Weller, T. H The Application of Tissue-Culture Methods to the Study of Poliomyelitis *New England J Med*, 249 186–195, 1953.
22. Scherer, W F, Syverton, J T, and Gey, G O Studies on the Propagation *in Vitro* of Poliomyelitis Viruses IV Viral Multiplication in a Stable Strain of Human Malignant Epithelial Cells (Strain HeLa) Derived from an Epidermoid Carcinoma of the Cervix *J Exper Med*, 97 695–710, 1953
23. Schubert, J H, Stanford, S M, and Tiffany, E J Comparative Evaluation of Several Complement Fixation Techniques for Laboratory Diagnosis of the Rickettsioses *J. Lab and Clin Med*, 37 388–393, 1951
24. Kaplan, M M, and Hulse, E C Prevalence of Q Fever in Europe and Survey Methods for Its Detection, in *World Health Organization Monograph Series*, No 19, 1953, pp 179–191
25. Collier, F C, Reich, A, and King, J W The Importance of the Sheep Cell Suspension in the Complement Fixation Reaction Description of an Accurate Technique for the Preparation of a Uniform Cellular Suspension *J Lab and Clin Med*, 39 778–784, 1952
26. Dean, H R, and Webb, R A The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction *J Path and Bact*, 29 473–492, 1926
27. Lighter, A. G Observations on Anticomplementary Reactions *Arch Dermat and Syph*, 67 362–368, 1953
28. Hilleman, M R, and Werner, J H Influence of Non Specific Inhibitor on the ...ienza *J Immunol*, 71 110–117, 1953
29. ... A A Study of Infections Caused by ... Specific and Non-Specific Serologic Rela-

30. Wenner, H. A., Monley, A., and Jenson, M H A Study of Infections Caused by Mumps and Newcastle Disease Viruses II Some Properties of Specific and Non-Specific Serum Hemagglutination Inhibitor Components *J Immunol*, 68 357–368, 1952

31. Verlinde, J D, and de Baan, P Sur l'hémagglutination par des virus poliomyélitiques murins et la destruction enzymatique des récepteurs de virus poliomyélitique de la cellule réceptive *Ann Inst Pasteur*, 77 632–641, 1949.

32 Yager, R H, Kuttler, K L, Olitsky, P K, and Murphy, L C. Hemagglutination by Neurotropic Viruses *U S Armed Forces M J*, 1 920–925, 1950.

33 Olitsky, P K, and Yager, R H Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses Experiments with Other Viruses *Proc Soc Exper Biol and Med*, 71 719–724, 1949

34 Chanock R M, and Sabin, A B The Hemagglutinin of St Louis Encephalitis Virus III Properties of Normal Inhibitors and Specific Antibody Use of Hemagglutination-Inhibition for Diagnosis of Infection *J Immunol*, 70 302–316, 1953

35 Casals, J, and Brown, L V Hemagglutination with Certain Arthropod-Borne Viruses *Proc Soc Exper Med and Biol*, 83 170–173, 1953

36 Gallagher, C D and Gallagher J R Effect of Aureomycin on the Development of Cold Agglutinins in Primary Atypical Pneumonia *New England J Med*, 247 314–316 1952

37 Keltel, H G Occurrence of Cold and Streptococcus MG Agglutinins in Infants with Gastroenteritis *J Inf Dis* 86 219–222, 1950

38 Burnet, F M, and Faris, D D The Technique of Quantitative Chorio Allantoic Virus Titration *J Bact*, 44 241–248, 1942

39 Jawetz, E and Coleman V R Studies on Herpes Simplex Virus III The Neutralization of Egg-Adapted Herpes Virus by Human Sera *in Ovo* *J Immunol*, 68 645–653, 1952.

40 Lennette, E H, and Koprowski, H Neutralization Tests with Certain Neurotropic Viruses A Comparison of the Sensitivity of the Extraneural and Intracerebral Routes of Inoculation for the Detection of Antibodies *J Immunol* 49 375–385, 1944.

41 Lennette E H and Koprowski H Comparative Sensitivity of the Extraneural and Intracerebral Neutralization Tests in Following the Antibody Response in Man to Vaccination with Western Equine Encephalomyelitis Virus *J Immunol*, 52 343–353, 1946

42 Crawley J F Neutralization Tests with Eastern Equine Encephalomyelitis Virus in the Chick Embryo A Statistical Treatment of the Results by the Probit Method *J Immunol* 59 319–330 1948

43 Sabin, A B The Dengue Group of Viruses and Its Family Relationships *Bact Rev*, 14 225–232, 1950

44 Smithburn, K Personal communication 1953

45 Tyrrell, D A J and Horsfall F L Jr Neutralization of Viruses by Homologous Immune Serum I Quantitative Studies on Factors Which Affect the Neutralization Reaction with Newcastle Disease Influenza A, and Bacterial Virus T *J Exper Med* 97 845–861 1953

46 Tyrrell D A J Neutralization of Viruses by Homologous Immune Serum II Theoretical Study of the Equilibrium State *J Exper Med*, 97 863–870 1953

47 Gaddum, J H Reports of Biological Standards III Methods of Biological Assay Depending on a Quantal Response Med. Res Council, London *Special Report Series*, No 183 1933

48 Reed L J and Muench H A Simple Method of Estimating Fifty Per Cent End points *Am J Hyg* 27 493–497 1938

49 Kärber G Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche *Arch f exper Path u Pharmakol* 162 480–483 1931

50 Bross I Estimates of the LD_{50} A Critique *Biometrics* 6 413–423 1950

7. Ginsberg, H S, and Horsfall, F L, Jr A Labile Component of Normal Serum

8.

9.

10.
Preservation of Viruses of the Psittacosis-Lymphogranuloma Venereum Group and Herpes Simplex Under Various Conditions of Storage *J Bact*, 63.369–376, 1952.

11 Horsfall, F L, Jr A Low Temperature Storage Cabinet for the Preservation of Viruses *J Bact*, 40 559–568, 1940.

12 Horsfall, F L, Jr, and Ginsberg, H S An Improved CO_2 Cabinet for Low Temperature Storage of Infectious Agents with Gaseous CO_2 Excluded from the Specimen Compartment *J Bact*, 61 443–451, 1951.

13 Van Rooyen, C. E, and Rhodes, A. J *Virus Diseases of Man*. 2d ed. New York, Thomas Nelson and Sons, 1948.

14 Rhodes, A J, and van Rooyen, C E *Textbook of Virology*. 2d ed. Baltimore, The Williams and Wilkins Company, 1953

15 Horsfall, F L, Jr Advances in the Diagnosis of Virus Infections. *Bull New York Acad Med*, 26 3–19, 1950

16 Lennette, E H, and Koprowski, H Influence of Age on the Susceptibility of Mice to Infection with Certain Neurotropic Viruses *J Immunol*, 49.175–191, 1944

17 Lennette, E H. Isolation of St. Louis Encephalitis Virus from a Fatal Human Case in California *Proc. Soc Exper Biol. and Med*, 61 206–210, 1946.

18 Cox, H R Growth of Viruses and Rickettsiae in the Developing Chick Embryo *Ann New York Acad Sc*, 55 236–247, 1952

19 Cottral, G E Endogenous Viruses in the Egg *Ann New York Acad Sc*, 55 221–235, 1952

20 Enders J F., Weller, T H, and Robbins, F. C Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues *Science*, 109 85–87, 1949

21 Weller, T. H The Application of Tissue-Culture Methods to the Study of Polio-

22

23 Schubert, J H, Stanford, S M, and Tiffany, E J Comparative Evaluation of Several Complement Fixation Techniques for Laboratory Diagnosis of the Rickettsioses *J Lab and Clin Med*, 37·388–393, 1951

24 Kaplan, M M, and Hulse, E C Prevalence of Q Fever in Europe and Survey Methods for Its Detection, in *World Health Organization Monograph Series*, No 19, 1953, pp 179–191.

25 Collier, F C, Reich, A, and King, J W The Importance of the Sheep Cell Suspension in the Complement Fixation Reaction Description of an Accurate Technique for the Preparation of a Uniform Cellular Suspension *J Lab and Clin Med*, 39 778–784, 1952

26. Dean, H R, and Webb, R A The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction *J Path and Bact*, 29 473–492, 1926

27. Lighter, A G Observations on Anticomplementary Reactions *Arch Dermat. and Syph*, 67 362–368, 1953

28 Hilleman, M R, and Werner, J H Influence of Non-Specific Inhibitor on the … a *J Immunol*, 71 110–117, 1953

29 … A Study of Infections Caused by … ific and Non-Specific Serologic Rela-

30 Wenner, H A, Monley, A, and Jenson, M H A Study of Infections Caused by Mumps and Newcastle Disease Viruses II Some Properties of Specific and Non-Specific Serum Hemagglutination Inhibitor Components *J Immunol*, 68 357–368, 1952.

31 Verlinde, J D, and de Baan, P Sur l'hémagglutination par des virus poliomyélitiques murins et la destruction enzymatique des récepteurs de virus poliomyélitique de la cellule réceptive. *Ann Inst Pasteur*, 77 632–641, 1949

32 Yager, R. H, Kuttler, K L, Olitsky P K, and Murphy L C Hemagglutination by Neurotropic Viruses *U S Armed Forces M J* 1 920–925, 1950

33 Olitsky P K., and Yager, R H Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses Experiments with Other Viruses *Proc Soc Exper Biol and Med*, 71 719–724, 1949

34 Ch

35 Ca

36. Gallagher, C D, and Gallagher J R Effect of Aureomycin on the Development of Cold Agglutinins in Primary Atypical Pneumonia. *New England J Med*, 247 314–316, 1952

37 Keitel, H G Occurrence of Cold and Streptococcus MG Agglutinins in Infants with Gastroenteritis *J Inf Dis* 86 219–222, 1950

38 Burnet, F M, and Faris, D D The Technique of Quantitative Chorio-Allantoic Virus Titration *J Bact* 44 241–248, 1942

39 Jawetz, E and Coleman V R Studies on Herpes Simplex Virus III The Neutralization of Egg-Adapted Herpes Virus by Human Sera *in Ovo* *J Immunol*, 68 645–653, 1952.

40 Lennette, E H and Koprowski H Neutralization Tests with Certain Neurotropic Viruses A Comparison of the Sensitivity of the Extraneural and Intracerebral Routes of Inoculation for the Detection of Antibodies *J Immunol* 49 375–385, 1944

41 Lennette E H and Koprowski H Comparative Sensitivity of the Extraneural and Intracerebral Neutralization Tests in Following the Antibody Response in Man to Vaccination with Western Equine Encephalomyelitis Virus *J Immunol*, 52 343–353, 1946

42 Crawley J F Neutralization Tests with Eastern Equine Encephalomyelitis Virus in the Chick Embryo A Statistical Treatment of the Results by the Probit Method *J Immunol* 59 319–330 1948

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44 Smithburn, K

45 Tyrrell, D A
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48 Reed L J and Muench H A Simple Method of Estimating Fifty Per Cent End-points *Am J Hyg* 27 493–497 1938

49 Kärber G Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche *Arch f exper Path u Pharmakol* 162 480–483 1931

50 Bross I Estimates of the LD$_{50}$ A Critique *Biometrics* 6 413–423, 1950

7. Ginsberg, H S, and Horsfall, F L, Jr A Labile Component of Normal Serum Which Combines with Various Viruses. Neutralization of Infectivity and Inhibition

8.

9

10 Allen, E G, Kaneda, B, Girardi, A J, Scott, T. F. McN., and Sigel, M. M. ... Group and ...-376, 1952.

11 ... servation of

12 Horsfall, F L, Jr, and Ginsberg, H S An Improved CO_2 Cabinet for Low Temperature Storage of Infectious Agents with Gaseous CO_2 Excluded from the Specimen Compartment *J Bact*, 61 443–451, 1951.

13 Van Rooyen, C E, and Rhodes, A. J. *Virus Diseases of Man* 2d ed. New York, Thomas Nelson and Sons, 1948.

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17 Lennette, E H. Isolation of St Louis Encephalitis Virus from a Fatal Human Case in California *Proc Soc Exper Biol and Med*, 61·206–210, 1946.

18 Cox, H R Growth of Viruses and Rickettsiae in the Developing Chick Embryo *Ann New York Acad Sc*, 55 236–247, 1952.

19 Cottral, G E Endogenous Viruses in the Egg *Ann New York Acad Sc*, 55 221–235, 1952

20 Enders, J F, Weller, T H, and Robbins, F C Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues. *Science*, 109 85–87, 1949

21. W...

22. Sc

23 Schubert, J H, Stanford, S M, and Tiffany, E J Comparative Evaluation of Several Complement Fixation Techniques for Laboratory Diagnosis of the Rickettsioses *J. Lab and Clin Med*, 37 388–393, 1951

24 Kaplan, M. M, and Hulse, E C Prevalence of Q Fever in Europe and Survey Methods for Its Detection, in *World Health Organization Monograph Series*, No 19, 1953, pp 179–191

25 Collier, F C, Reich, A, and King, J W The Importance of the Sheep Cell Suspension in the Complement Fixation Reaction Description of an Accurate Technique for the Preparation of a Uniform Cellular Suspension *J Lab and Clin Med*, 39·778–784, 1952

26 Dean, H R, and Webb, R A The Influence of Optimal Proportions of Antigen and Antibody in the Serum Precipitation Reaction *J. Path and Bact*, 29 473–492, 1926

27 Lighter, A G Observations on Anticomplementary Reactions *Arch Dermat. and Syph*, 67·362–368, 1953

28 Hilleman, M R, and Werner, J H Influence of Non-Specific Inhibitor on the ... *J Immunol*, 71 110–117, 1953

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33 Olitsky, P K, and Yager, R H Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses Experiments with Other Viruses *Proc Soc Exper Biol and Med*, 71 719–724, 1949

34 Chanock, R M, and Sabin, A B The Hemagglutinin of St Louis Encephalitis Virus III Properties of Normal Inhibitors and Specific Antibody Use of Hemagglutination-Inhibition for Diagnosis of Infection *J Immunol*, 70 302–316, 1953

35 Casals, J, and Brown, L V Hemagglutination with Certain Arthropod-Borne Viruses *Proc Soc Exper Med and Biol*, 83 170–173, 1953

36 Gallagher, C D, and Gallagher J R Effect of Aureomycin on the Development of Cold Agglutinins in Primary Atypical Pneumonia *New England J Med*, 247 314–316 1952

37 Kettel, H G Occurrence of Cold and Streptococcus MG Agglutinins in Infants with Gastroenteritis *J Inf Dis*, 86 219–222, 1950

38 Burnet F M, and Faris, D D The Technique of Quantitative Chorio Allantoic Virus Titration *J Bact*, 44 241–248, 1942

39 Jawetz, E, and Coleman V R Studies on Herpes Simplex Virus III The Neutralization of Egg-Adapted Herpes Virus by Human Sera *in Ovo J Immunol*, 68 645–653, 1952.

40 Lennette, E H, and Koprowski, H Neutralization Tests with Certain Neurotropic Viruses A Comparison of the Sensitivity of the Extraneural and Intracerebral Routes of Inoculation for the Detection of Antibodies *J Immunol* 49 375–385, 1944

41 Lennette E H and Koprowski H Comparative Sensitivity of the Extraneural and Intracerebral Neutralization Tests in Following the Antibody Response in Man to Vaccination with Western Equine Encephalomyelitis Virus *J Immunol*, 52 343–353, 1946

42 Crawley J F Neutralization Tests with Eastern Equine Encephalomyelitis Virus in the Chick Embryo A Statistical Treatment of the Results by the Probit Method *J Immunol* 59 319–330 1948

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44 Smithburn K Personal communication 1953

45 Tyrrell, D A J and Horsfall F I Jr Neutralization of Viruses by Homologous Immune Serum I Quantitative Studies on Factors Which Affect the Neutralization Reaction with Newcastle Disease Influenza A, and Bacterial Virus T *J Exper Med*, 97 845–861 1953

46 Tyrrell, D A J Neutralization of Viruses by Homologous Immune Serum II Theoretical Study of the Equilibrium State *J Exper Med* 97 863–870 1953

47 Gaddum J H Reports of Biological Standards III Methods of Biological Assay Depending on a Quantal Response Med Res Council, London Special Report Series No 183 1933

48 Reed L J, and Muench H A Simple Method of Estimating Fifty Per Cent End points *Am J Hyg* 27 493–497 1938

49 Kärber, G Beitrag zur kollecktiven Behandlung pharmakologischer Reihenversuche *Arch f exper Path u Pharmakol* 162 480–483 1931

50 Pross I Estimates of the LD_{50} A Critique *Biometrics* 6 413–423 1950

STATE OF CALIFORNIA DEPARTMENT OF PUBLIC HEALTH DIVISION OF LABORATORIES
VIRAL AND RICKETTSIAL DISEASE LABORATORY
1392 UNIVERSITY AVENUE BERKELEY 2 CALIFORNIA

VIRAL AND RICKETTSIAL DISEASE SPECIMEN HISTORY

PLEASE FILL OUT THIS HISTORY IN FULL AND SEND TO LABORATORY WITH SPECIMEN

Name	Age	Sex
Address (Street and City)	Occupation	
DISEASE SUSPECTED	DATE OF ONSET	Kind of specimen

Specimen ☐ 1st ☐ 2nd ☐ 3rd | Dates taken 1st ____ 2nd ____ 3rd ____

Physician	Address (Street and City)

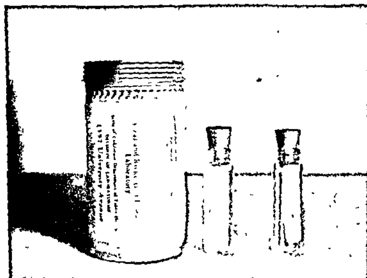
Clinical remarks:

Rev. 3-49
Form VL 14

SEE REVERSE FOR IMPORTANT INSTRUCTIONS

Photograph by California State Department of Public Health

FIG. 1. Example of form providing minimal data required on specimens for examination to be submitted to laboratory with specimen.



Photograph by California State Department of Public Health

FIG. 2. Self-addressed container with sterile vials, for mailing blood specimens.

POLIOMYELITIS

JOHN R. PAUL, M.D.
Professor of Preventive Medicine, Yale University School of Medicine, New Haven, Connecticut

and

JOSEPH L. MELNICK, PH D
Professor of Epidemiology, Yale University School of Medicine, New Haven, Connecticut

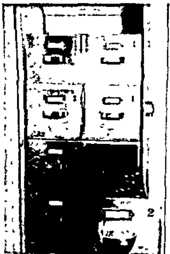


Fig 3 Method for filing serum specimens in chronologic sequence and by accession numbers Units fabricated of stainless steel to obviate corrosion from rust



Fig 4 Dry-ice cabinet with slide units containing drawers for storage and filing of specimens.



Fig 5 Upright deep freeze unit (—20° C) with compartmented drawers for storage of specimens Size of compartments adjustable through use of dividers in slots

attack, the so-called "minor" illness These asymptomatic infections and minor illnesses are of importance in the understanding of the epidemiology and immunology of poliomyelitis.

The main features of the various manifestations of poliomyelitis infection are as follows.

1 *Inapparent infection* Associates of cases of poliomyelitis are frequently infected without showing any clinical evidence of this process, and to a lesser degree this is true of other juvenile members of the community. Such infections are known as "inapparent," "asymptomatic," or "silent" and can be recognized only in the laboratory by the recovery of virus or the demonstration of an increase in serum antibody

2. *Clinical forms of poliomyelitis*—a *Abortive poliomyelitis* The clinical manifestations of abortive infections or minor illnesses include fever, headache, sore throat, listlessness, anorexia, and vomiting These illnesses usually last for only 24 to 48 hours and are commoner in children than in adults Abortive poliomyelitis cannot be diagnosed with certainty by clinical methods A tentative diagnosis can, however, be made on those patients who have been in close association with a more severe case of poliomyelitis For a positive diagnosis, laboratory methods are almost essential

b *Nonparalytic poliomyelitis* This term refers to a more severe illness in which definite manifestations of central nervous system involvement are added to those of the minor illness The symptoms indicate that the virus has produced lesions within the central nervous system to a degree that they are clinically detectable Clinical features include fever, headache, vomiting, pains in the back, neck, trunk, or limbs, paresthesias, and stiffness of the neck or back. There is usually an increase of cells and protein in the cerebrospinal fluid The clinical features are similar to those of *benign aseptic meningitis*, which may be due to a variety of causes, among which is the poliomyelitis virus

These are the manifestations of what is now usually called the "major" illness of poliomyelitis, a term also used for the paralytic cases In some cases, especially in children, illness may occur in two phases, the minor followed by the major illness, this is the so-called "dromedary" type of poliomyelitis, in which a few days of well-being separate two bouts of fever, of about 1 to 2 and 5 to 10 days, respectively.

The lumbar puncture is of supplementary diagnostic value in cases of nonparalytic poliomyelitis, but it may not be possible to make an accurate diagnosis without resort to virologic or immunologic tests

c *Spinal paralytic poliomyelitis* The major illness of poliomyelitis is one which may clear up completely within 4 to 7 days without paralysis, or it may become more severe and develop into the classical form of paralytic poliomyelitis. Paralysis usually develops after 1 to 4 days of the symptoms just described. The muscles commonly paralyzed, in order of frequency, are those of the legs, arms, back, thorax, *intercostal region, and diaphragm Paralysis of the muscles of respiration* may occur

d *Bulbar paralytic poliomyelitis* In this variety of poliomyelitis, there is paralysis of one or more muscle groups innervated by the cranial nerves, especially those of the soft palate and pharynx, and this gives rise to dysphagia, dyspnea, nasal speech, paralysis of the muscles of the face, tongue, jaw, and eye. Paralysis

I INTRODUCTION

Poliomyelitis (infantile paralysis) is a common acute infectious disease more often seen in children than in adults, which offers serious problems to the public health officer. It is likely to appear in epidemic form, particularly in the summertime Clinically, it is characterized by sudden onset, with a short period of fever, headache, and vomiting, and this is the extent of the clinical picture in the great majority of cases. On the other hand, in an appreciable fraction of the cases, clinical evidence of myelitis occurs, often resulting in flaccid paralysis of various groups of muscles.

A. Clinical and Epidemiologic Considerations

It must be strongly emphasized that *paralysis* is an infrequent complication of poliomyelitis infection in man and that most persons who become infected either show no symptoms or develop a transient abortive

week of the disease In the intestinal tract its detection during the first 2 or 3 weeks of convalescence is commonplace, and it may remain there until the 12th week from onset or longer Nevertheless, it is suspected that the amount of virus excreted per day by the patient is much greater in the early stages (1st week) than later. The extent of the clinical symptoms, or the degree of paralysis which a given patient shows, has little bearing on the amount of virus which may be present in the throat or the intestinal tract, for mild, abortive cases harbor the virus in these sites as readily as do the paralytic cases In fact, since the symptoms may be insignificant, many so-called "healthy" carriers may actually be convalescent carriers As a rule, within the same epidemic area, mild cases and "healthy" carriers represent the same age group as that which one sees among paralytic patients, namely, children varying in age from 3 to 18 and particularly children from age 6 to 12 * The part which mild cases or carriers, both convalescent and apparently healthy, may play in the general spread of the disease is unknown, but it is probably considerable

7. *Dissemination of the virus* Poliomyelitis viruses are agents highly infectious for young children, which can probably be transmitted by a variety of methods All the evidence points to the fact that the most important means of spread is by means of pharyngeal and fecal excretions of infected human individuals As a result, poliomyelitis can probably be acquired by association with an infected person of such an intimate nature that direct transfer of virus to the alimentary tract of the susceptible person is made possible Under such circumstances, the importance of virus in pharyngeal excretions is evident

Feces provide a rich and persistent source of virus As much as 1 million infectious doses for monkeys can be detected in a gram, although smaller concentrations of virus are more often found It seems probable that poliomyelitis virus can be as readily disseminated by fecal contamination as is bacillary dysentery Since opportunities for the direct transfer of fecal contamination to the mouth are very frequent, it is readily understood that infection with poliomyelitis virus is easily acquired

Sewage contains large amounts of virus when infection occurs within the community served Polluted water used for drinking, bathing, and washing is therefore a risk Milk, drinking water, or foodstuffs which are contaminated with virus constitute potential vehicles of transfer, but here again actual demonstrations that the disease is spread in this way have been few Nevertheless, the fact that infection is acquired early in life, and is so uniform in crowded areas and communities with poor sanitation and much fecal pollution of the environment, supports the likelihood that these materials are important and major sources of distribution of virus Conversely, in areas with less crowding and better sanitation, exposure and infection are reduced so that the dissemination of virus is more sharply limited

The role of flies in the dissemination of poliomyelitis is not clear, but they are certainly not essential, and there is no evidence that virus multiplies within flies That flies can become readily contaminated with virus from infected feces and may

* Age groups vary in different parts of the world, and the figures given above apply to the northern half of the United States and to most of Europe In New England, as of 1953, almost 30 per cent of the cases are older than 15 years, whereas in Cairo, Egypt, 95 per cent of the cases in the local inhabitants occur in infants below the age of 5 years.

of the circulatory and respiratory centers may develop with great rapidity and are of serious prognosis. Practically all the deaths from poliomyelitis are attributable to bulbar involvement.

e *Encephalitic manifestations* These may appear in association with any of the other forms of the disease. Symptoms include mental clouding, coma, and tremors.

3. *Incubation period* This is usually said to be about 10 days, but it is probably shorter, 4 to 6 days, particularly when the earliest signs of illness are chosen to mark the onset of the disease. The range extends from 4 to 35 days

4. *Changes in the cerebrospinal fluid* The cerebrospinal fluid does not yield the virus in the acute or other stages, but its examination for other properties affords valuable confirmation of the clinical diagnosis, that is, by the detection of an increased number of cells and of protein. In the *abortive* type or in the minor illness the spinal fluid is apt to be negative, but in the early stages of the major illness, whether it be nonparalytic or paralytic, there is likely to be an increase in the cell count, which may range from 10 cells to over 500 per cmm. In the early stages, up to 85 per cent of these cells may be neutrophils. With the progress of the illness, the cell count falls and lymphocytes predominate. The protein content is often elevated in early stages of the illness (that is, above 40 mg %), and may continue to rise (to 500 mg. % or higher) for 2 to 3 weeks, even in the face of a normal cell count. It is well to recall that some 10 per cent of the paralytic cases have a negative spinal fluid

5. *Differential diagnosis* The nonparalytic illness may be diagnosed tentatively on the results of clinical examination, suitable tests on the cerebrospinal fluid (see above), and a history of association with a paralytic case of poliomyelitis It must be realized, however, that many other agents cause an *aseptic meningitis* that cannot be differentiated from nonparalytic poliomyelitis, except by special laboratory tests that are only available in a limited number of virus laboratories The commonest viruses causing aseptic meningitis simulating poliomyelitis are the following *mumps, L C M, echo, herpes simplex, herpes zoster,* and *epidemic encephalitis.* Certain Group B *Coxsackie viruses* have been incriminated by various workers. A considerable number of cases of aseptic meningitis caused by *leptospira* have been reported In addition, in the initial stages of illness it may be difficult to differentiate *tuberculous* or *pyogenic meningitis* from aseptic meningitis caused by viruses

6 *Pathogenesis.* Theoretically, the virus of poliomyelitis usually enters the human body through a number of possible portals, including the mucosa of the oral cavity, the upper or lower gastrointestinal tract, or conceivably the skin. It seldom gains access to the central nervous system solely and directly by way of the nasal mucosa and olfactory bulbs Once in the body, the virus shows affinity for three areas in particular (*a*) the intestinal tract (particularly the lower ileum), where it may survive for weeks, (*b*) the mouth or pharynx, where it survives for a shorter period; and (*c*) certain areas of the central nervous system, where it does the greatest harm. The virus has been demonstrated in the blood during the incubation period and during the minor illness, but whether the blood stream is actually one of the paths of transmission from the alimentary tract to the central nervous system is not known. The virus, however, seldom remains in the blood for longer than a few days In the buccal cavity or pharynx it seldom persists longer than the 1st

the viruses. In the electron microscope the virus appears as spherical particles of 30 mμ diameter In purified preparations from tissue culture fluids they tend to form crystal-like arrays.

b Resistance to physical and chemical agents In contrast to many bacteria, poliomyelitis virus is quite stable if kept moist and cold. It remains viable at icebox temperatures in aqueous suspensions of feces for months, and similarly in pieces of infected spinal cord stored in 50 per cent glycerol for years On the other hand, poliomyelitis virus seems to be highly sensitive to complete desiccation even in the presence of protein, glucose, or other similar preservatives, and even when dried from the frozen state Resistance to heat is influenced by the nature of the suspending medium Thus, milk and cream exert a marked protective influence Even under conditions of optimal stability, however, temperatures above 60° C cause rapid inactivation

Application of heat is probably the most practicable means of disinfection. Ultraviolet light cannot be relied upon to disinfect material contaminated with poliomyelitis virus

Resistance to chemical agents on the other hand is remarkable Optimal stability is about pH 8, but rapid inactivation is not observed until below pH 2 or above pH 10 Of organic solvents, ether is without destructive effect, alcohol and acetone inactivate the virus slowly, but more rapidly at higher temperatures Such disinfectants as phenol and formalin inactivate but slowly, and comparatively high concentrations are needed However, sterilization of such delicate instruments as bronchoscopes and catheters in formaldehyde vapor at 50° C. seems to be practicable Oxidizing agents such as hydrogen peroxide and potassium permanganate seem to be active chemical disinfectants. 0.05 p.p.m. of *free* chlorine is sufficient for complete inactivation of relatively pure virus in 15 minutes, iodine acts even faster. It must be pointed out, however, that the critical concentration of all chemical agents is largely dependent upon the medium in which they have to exert their action Presence of organic material, in particular protein or degradation products thereof, provides a considerable protection against inactivation and, for this reason, no standard concentrations can be mentioned. Virus present in the stools of patients has been found to resist treatment with chloride of lime as recommended for disinfection of stools from carriers of typhoid bacilli.

3. *Host range* A characteristic property of freshly isolated strains of poliomyelitis viruses is their narrow host range. With Types I and III, primates are the only animals which can be readily infected, whereas

act for 1 to 2 weeks as mechanical carriers has, however, been adequately demonstrated. It has been suggested that in many areas, especially the more temperate zones, the housefly is not commonly involved, but rather the sarcophagids and blowflies. On the other hand, where fresh excreta are a significant source of moisture, flies of the genus *Musca* will also feed They can ingest virus or can be contaminated with virus and theoretically transfer it for consumption by a susceptible subject. Virus can also be recovered from cockroaches but, up to the present, biting insects have not been implicated.

The possibility may be considered that infected excretions introduced into wounds, or introduced by injections made through contaminated skin, can be a mode of infection

B. Characteristics of the Virus

1. *Types of virus.* Three immunologic types of human poliomyelitis virus have been identified, the prototype strains of which are: *Brunhilde* (Type 1); *Lansing* (Type 2); and *Leon* (Type 3) Strains are identified as of a certain type by appropriate neutralization tests with known antisera and by their ability to induce the formation of type-specific antibodies. The types seem quite distinct, and overlapping is usually not observed This is true with hyperimmune monkey sera used in neutralizing and complement fixation tests. When convalescent human sera have been allowed to react with concentrated antigens, however, the complement fixation test has revealed the existence of certain group antigens

The majority of observations have indicated that outbreaks of epidemic proportions have been attributable primarily to Type 1 strains Epidemics caused by Type 3 virus, however, have recently been described. Type 2, although sometimes found in epidemics, has usually been associated with sporadic cases.

2 *Physical and chemical properties* None of the characteristics here to be described is strictly specific. Therefore, it is at present not possible to identify a poliomyelitis virus by physical and chemical examination alone. If, on the other hand, an unknown agent differs significantly from the typical pattern, its classification as a poliomyelitis virus must be considered as doubtful.

a. Size and morphology. Information concerning these properties can be obtained by electron micrography, filtration end point, and sedimentation-diffusion rate determinations. For practical purposes, with new strains which have not been concentrated and purified, the filtra- [illegible] membranes seems

[illegible]

diameter) of about 35 mμ or less This marks it as one of the smallest of

b Intestinal contents and intestinal wall.

In removing central nervous system tissue at autopsy, it is well to have an assistant ready with sterile gloves and several sets of sterile instruments or at least with facilities for reboiling the same instruments frequently.* Favored sites for virus are· the medulla and cervical and lumbar sections of the spinal cord, material from these areas can be placed in a sterile petri dish A convenient size for these pieces is 2 cc. The cauda equina is not recommended as a source of virus Other appropriate sections of the cord may be placed simultaneously in fixing solution for subsequent histologic study

2 *Clinical cases and carriers.* Poliomyelitis virus has been isolated frequently from·

a. Feces
b. Rectal swabs; some fecal material should be obtained on the swab, if possible
c Pharyngeal (oro- or nasopharyngeal) washings
d. Throat swabs

A common, reliable source for virus is human fecal material because poliomyelitis virus seems to remain longer in the intestinal tract than elsewhere within the body Thus, poliomyelitis virus has been isolated from feces during the incubation period, the active disease, and convalescence Nevertheless, the optimal time for collecting specimens is early in the course of the infection. This applies to paralytic, nonparalytic, and abortive cases Virus may also be recovered from persons suffering from inapparent infections Such persons are often present in association (in both time and place) with known cases of poliomyelitis

The virus is usually not found in spinal fluid but has been detected in the blood during the incubation period and during the "minor illness" or first phase Blood is not a favored material for isolation, however.

C. Collection of Material from Patients and Carriers

1 *Feces* A 15- to 25-gm specimen is desirable Small wide-mouthed jars or heavy paper cartons are useful as containers The specimens should be kept frozen or at least kept cold until tested.

2 *Rectal swabs* If a stool specimen cannot be obtained, a rectal swab is often a useful substitute However, the chances of isolating virus from a swab are less than they are from a fecal specimen. To obtain the

* The main reasons for maintaining sterile technic is that if the central nervous system tissue is to be used immediately (or if it is to be kept in the frozen state) for intracerebral monkey inoculation or for tissue culture inoculation, the chances of bacterial contamination are minimized

with certain Type II strains, rodents (cotton rats, hamsters, and adult and suckling mice) can be infected as well. The adaptation of special strains of Types I and III to mice has, however, been recently accomplished although this procedure has not been easily done.

The growth of poliomyelitis viruses in tissue cultures is considered in a separate chapter.

II. ISOLATION AND IDENTIFICATION OF POLIOMYELITIS VIRUS

The directions covered in the following sections (II, A to F) can be applied to material to be used either for the inoculation of *animals* or *tissue cultures*. Section G is concerned largely with the technics of animal inoculation and Section H with those of tissue culture.

A. Precautions

Isolation of poliomyelitis virus from human and other sources usually requires a team of 2 or more workers, and their work is not without danger, for the percentage of laboratory workers who have accidentally acquired poliomyelitis within the laboratory is appreciable. For obvious reasons, therefore, the use of sterile technics in handling infective materials is mandatory. Gowns are recommended, and, for certain procedures, gloves as well. Persistent vigilance is necessary on the part of the director of the team engaged in this work if the individual members are to follow consistently the recommended sterile technics.

B. Sources of Material

Poliomyelitis virus can be isolated by *monkey* inoculation and by *tissue culture* inoculation, using materials from man and such extrahuman sources as sewage and flies. Human fecal material provides the richest and most available source. Even if the most careful technic is followed, however, it is a common experience to encounter negative results. If human material is being tested, the chances of a successful isolation are greater if the material is obtained early in the disease (within the first 7 to 8 days), dating the onset of the disease from the [illegible] that is, from the beginning of "minor

infection.

1. *Human autopsy material* from which the virus has been frequently isolated includes:
   a. Spinal cord, medulla, and pons, provided death of the patient has occurred within 7 to 10 days from the onset.

b) Do not allow the tissue to remain untested any longer than is necessary.

This last statement might be qualified with the comment that, as 50 per cent glycerol acts as a slow bactericidal agent, there is some virtue in allowing bacteriologically contaminated specimens to remain in it for a few days before they are inoculated. Although poliomyelitis virus has been known to survive in 50 per cent glycerol for many years, it has also died out in this medium after a few months or even weeks.

3. *Lyophilization.* Poliomyelitis virus is usually destroyed by drying, even from the frozen state. Although lyophilization as a method of preserving poliomyelitis has been accomplished in the presence of certain stabilizers, (*a*) 10 per cent monkey sera and (*b*) mucin, the results are irregular, and the method is not recommended for this purpose

E. Shipping of Specimens

If frozen material is to be shipped short distances, it should be sent in a proper container, this may be an insulated box containing dry ice or within a well-packed thermos flask containing dry ice Special care in packing thermos bottles is essential, or breakage may easily occur. The necessity of keeping *all* specimens cool probably varies with the circumstances.*

More practical for shipping is the use of 50 per cent glycerol. Autopsy specimens, stool specimens (when small in amount), and the sediment from rectal swabs, oropharyngeal washings, and throat swabs may all be sent at room temperature in 50 per cent glycerol For these it is convenient to use small, wide-mouthed bottles with tightly stoppered or capped orifices and, for safety's sake, the top of each bottle may be wrapped with several layers of waterproof tape Before preparing such material for inoculation the fragments of tissue or particulate matter should be washed several times in saline solution to remove some of the glycerol

F Preparation of Materials for Inoculation

1. *Nervous tissue* Weighed fragments of medulla or spinal cord (1 to 2 gm. are usually sufficient) are placed in a sterile mortar containing a small amount of sterile abrasive (alundum or sand), and this material is then ground with enough sterile water, not more than 1 or 2 ml. at first, to make it into a fairly thick paste. Grinding is usually

* Actually, the survival time of poliomyelitis virus in human stools at room temperature is unknown, but the virus does remain viable in this medium for several weeks at refrigerator temperature (0–4°)

best results a moist, sterile swab is inserted well into the rectum and rubbed about until fecal material is shown to be adhering to it. The swab is then replaced in a test tube containing 1 ml. of sterile water or broth It is important that the specimen be tested promptly, or kept frozen or in glycerol.

3. *Pharyngeal (or nasopharyngeal) washings.* Various types of irrigating fluid such as sterile distilled water or broth may be used to obtain nasopharyngeal washings from patients or suspected carriers of the virus. The irrigating fluid is introduced into the patient's mouth, either from a drinking glass or through a large glass syringe without a needle attached. The patient is then encouraged to gargle the material, which can be collected in a sterile drinking glass or small basin The procedure is carried on over a period of at least 3 minutes, using the same fluid repeatedly. The amount of washings is kept under 30 ml.

4. *Throat swabs.* Material is obtained by rubbing the oropharynx vigorously with 2 sterile cotton swabs, which are immediately transferred to a test tube containing 1 to 2 ml of sterile water or broth. The specimens should be tested promptly or kept frozen or in glycerol.

D. STORAGE OF MATERIAL

Material awaiting testing or shipment may be held for short periods at refrigerator temperature: 0–4° C. For longer storage, it should be frozen or kept in 50 per cent glycerol

1. *Freezing. Ordinary glass test tubes that contain more than 1 ml.* of fluid are liable to crack when frozen, so, if fluid material is to be frozen, it should be placed in a special container, that is, either in nitrocellulose tubes or thick-walled glass containers. Freezing is accomplished by placing the tubes in the freezing compartment of an electrically driven refrigerator, or in a specially constructed insulated box containing dry ice (carbon dioxide snow), which may maintain a temperature of from –20° to –70° C. For the preservation of poliomyelitis virus, unlike certain other viruses, temperatures below –20° C are not required

2. *Glycerol.* This is a time-honored, simple, and quite practical method of storing tissues or material containing poliomyelitis virus. *Only* the purest brands of glycerol available are desirable for the preservation of poliomyelitis virus. The glycerol should be mixed with an equal volume of physiologic saline solution before being used.

Some points with regard to the use of glycerol are.

a) Do not put more than 4 or 5 small pieces of tissue in 50 ml.

added as a bactericidal agent* together with penicillin and streptomycin solution to make a final dilution of 500 units and 500 μg per ml, respectively. The etherized suspension is kept in a stoppered container in the refrigerator.

A sample of Part II (5 to 10 ml) is placed in a small centrifuge tube within the centrifuge cup in the icebox to chill the specimen. In order to remove bacteria, it is again centrifuged at 4,500 r p m., or at speeds up to 18,000 r p m. (forces about 20,000 times gravity), if such are available, for ½ to 1 hour. From the supernate of this specimen, 3 ml. are drawn off and, of this, 0 1 ml. is cultured on a blood agar plate This specimen, Part III, is employed for inoculation of tissue cultures, the dose being 0.1 to 2 0 ml. per culture. If it is to be used for intracerebral (i c.) inoculation of monkeys, it is set aside in the refrigerator If the growth of bacteria (after 24 hours) in Part III is minimal or absent, the fluid below the layer of ether is removed and is then inoculated intracerebrally, in an amount not exceeding 1 ml, or intraspinally with not more than 0.2 ml

The same monkey may be inoculated intra-abdominally with the residuum of Part II—using an inoculum of not more than 10 to 12 ml The concentration of ether (15 per cent) and antibiotics used in Part II is usually but not always sufficient to destroy or diminish the number of bacteria in the suspension to permit the intra-abdominal injection of 10 to 12 ml without fear of inducing fatal peritonitis.

Rectal swabs If only the washings from a rectal swab are available, the volume of material will be small, but can be diluted up to 3 to 5 ml The cotton swab should be transferred aseptically to the barrel of a sterile 1 ml. syringe, and 2 or 3 ml of diluent added (buffer at pH 8) The piston of the syringe is now inserted into the barrel and the virus eluted from the cotton by pressing the piston firmly into the barrel. Elution can be made complete by taking the diluent into the syringe and expressing it again in several cycles Antibiotics are added, and the suspension is otherwise treated as in Parts II and III and used for inoculation of tissue cultures or intracerebral inoculation of monkeys.

Concentration of virus by ultracentrifugation The virus contained in fecal suspensions can be concentrated and separated from much extraneous material by the use of the ultracentrifuge[1] A larger amount of fecal suspension is prepared as for Part III above, and 10 to 100 ml., depending upon the availability and degree of concentration desired, are spun in the ultracentrifuge for 60 minutes at 39,000 r.p.m. (106,000 times gravity) The supernate is discarded and the virus-containing

* If the material is to be inoculated into tissue culture the addition of ether can be omitted.

done for at least 5 minutes. Sufficient cold, sterile, distilled water is then added to make a 10 per cent suspension, and to the supernatant fluid antibiotics are added to yield a final concentration of 500 units of penicillin per ml. and 500 μg. of streptomycin per ml. The suspension is then transferred to a cold centrifuge tube, where it is spun at low speed (2,000 r.p m) for 5 minutes The supernatant fluid will be opalescent but should contain no particles large enough to plug the lumen of a small needle. It is advisable to make up from 10 to 15 ml of suspension so that some of the material may be kept frozen for possible future use.

2. *Feces and rectal swabs.* Relatively large (25 to 50 gm) specimens are sometimes desirable but not essential. In preparing this material for inoculation, various procedures may be used, none of them is foolproof, and any laboratory that is beginning to do work in this field may expect to encounter difficulties which may be overcome through a process of trial and error.

Primarily, it may be desirable to divide the original specimen of feces (or fluid from the rectal swab) in half, the 2d half being kept frozen or in the icebox for future use, in case the test is unsatisfactory or in case there are other reasons to retest the specimen

The procedures are designed to kill or remove bacteria from the material containing the virus The extent of treatment is determined by whether the material is to be inoculated into tissue culture or into monkeys and, if the latter, by what routes Another factor to consider is whether the virus-containing fraction should be concentrated prior to inoculation so as to increase the chances for isolation.

A method which has given satisfactory results is as follows. a 10 or 20 per cent suspension is made from the stool specimen in cold, sterile, distilled water in a tightly stoppered 250 ml. flask containing glass beads,* after frequent shaking, the specimen is allowed to settle in the cold. The supernatant fluid is then poured off into another stoppered flask, and it is again well shaken and allowed to settle From the supernate of the second flask, the material is divided into two parts, I and II, generally amounting to between 20 and 25 ml each

Part I (20 ml , untreated with ether) is kept in reserve at icebox temperature.

Part II (25 ml) is immediately centrifuged at relatively low speed (15 minutes, 2,000 r p m) and, to the supernate, 15 per cent ether is

* The addition of 20 mg of phenol red per l of water is recommended The indicator designates those specimens which may be cytotoxic for tissue cultures because of extraordinary pH values

Fig 1). When kept in small cages, however, they should be let out and exercised daily. Monkeys confined within small cages for any length of time without adequate exercise often develop stiffness and atrophy of the limbs—so-called cage paralysis More than once this cage paralysis has been mistaken by inexperienced workers and others for experimental poliomyelitis

Understandable objections have been raised to the practice of allowing inoculated monkeys to exercise in a common area between small cages, because of the possible chances of cross-infection between animals This objection may be justified in the case of cynomolgus monkeys and chimpanzees, for with the chimpanzee, in particular, infection can be easily induced by oral administration of the virus, and it can be acquired accidentally in the laboratory But over a period of 40 some years of poliomyelitis research in which it has been common practice to allow more than one rhesus monkey per cage, spontaneous infections of this species with poliomyelitis virus seem to have occurred with rarity

On arrival in the laboratory, new monkeys, fresh from the dealers, should be placed in special cages for observation Those in poor condition should be isolated or eliminated, especially those with diarrhea, skin diseases, malnutrition, and cough. It is always advisable to observe monkeys for a period of about 10 days prior to their inoculation During this period a certain amount of conditioning of the animal can be practiced, and those which are too far gone can be sacrificed Weak and sick animals suffering from malnutrition, diarrhea, etc., sometimes make a remarkable (but slow) recovery if properly isolated and given antibiotics and a good diet

b Tuberculin testing If the animals have not been tuberculin-tested before their arrival in the laboratory, it is good practice to carry out this test This is done according to Schroeder's test, with 1 mg O T (old tuberculin), using the upper or lower eyelid as the site of the inoculation Monkeys with positive evidences of tuberculosis should be eliminated immediately for at least three reasons (a) they may die within 5 weeks, (b) they may spread the disease to other members of the monkey colony, and (c) they are a potential source of danger to laboratory personnel since they are often infected with human types of tubercle bacilli

c Diet The diet used in our laboratories consists mainly of crackers specially designed for feeding animals We have used Purina chow, but these small pellets fall through the false bottom of the cage where they are either lost or become contaminated with excreta The "chimcracker" or "Lifespan" dog biscuits, because of their larger size (approximately 3 by 2 by ¼ inches), have proved more satisfactory Some of the satisfactory monkey diets are listed below

Satisfactory Diets for Monkeys

1 Supplied by The Kennel Food Supply Co, Fairfield, Connecticut

The chimcracker formula contains twelve ingredients and consists of 2d clear wheat flour, 40.8 per cent, soybean meal, 16 5 per cent, cornmeal, 8 7 per cent, ground wheat, 4 85 per cent, powdered skimmed milk, 4.85 per cent, ground raisins, 4.85 per cent, bone meal, 4.85 per cent, salad oil, 4.85 per cent, molasses (blackstrap), 3 9 per cent, salt, 6 per cent, wheat germ, 4.3 per cent, irradiated yeast, 1 0 per cent A sample analysis yielded the following proteins, 21.5 per cent; fats, 6 9 per cent, carbohydrates, 52 1 per cent, ash, 6 9 per cent, phosphorus, 0 98 per cent, calcium, 1.86 per cent, and iron, 0.035 per cent.

2 Supplied by Lifespan National Canine Products, 1133 McDonald Ave., Brooklyn 30, New York.

The composition of the biscuit is similar to "chimcracker"

sediment is taken up in 1 to 3 ml of sterile water. This suspension is prepared for inoculation by centrifugation for 30 minutes at 4,500 r p.m. (or 20 minutes at 18,000 r.p m) to remove extraneous debris in the sediment, and antibiotic added as above. While this method is more delicate and valuable for special studies, its use is not mandatory, and therefore an ultracentrifuge is not considered an essential part of the usual poliomyelitis laboratory's equipment

3. *Pharyngeal washings and throat swabs.* The washings are transferred to a sterile flask containing glass beads, the flask is tightly stoppered and shaken for 10 minutes If tissue cultures are to be inoculated, the suspension is subjected to centrifugation at 4,500 to 18,000 r p.m. for ½ to 1 hour and antibiotics added as indicated above. The inoculum is 0 1 to 2 0 ml. per culture.

If monkeys are to be inoculated, the original suspension after a preliminary shaking is subjected to light centrifugation, 2,000 r p m. for 10 minutes, 10 per cent ether and antibiotics are added to the supernate, and the suspension is allowed to stand in the refrigerator overnight. On the following day, the treated material is inoculated intracerebrally in 1 ml amounts. The intracerebral inoculation may be supplemented by an intra-abdominal injection of 10 ml of the etherized suspension.

Throat swabs are treated in the same way as outlined for rectal swabs.

G Monkey Inoculation

1 *Species* The following species of monkeys have been most often used in poliomyelitis work:

Macaca mulatta the rhesus monkey, usually from India

Macaca cynomolgus and/or *irus* or *mordax* the cynomolgus (or Java) monkey from the East Indies, Philippine Islands, or Malaya

Cercopithecus aethiops sabaeus and *griseoviridis:* the green African and grivet monkeys from West and East Africa.

Cercopithecus aethiops centralis the vervet monkey from West, Central, and South Africa

Cebus capucina the ringtail or capuchin monkey from South America

Of this series the rhesus monkey has been most widely used One, 2, or 3 monkeys may be used for testing each specimen It is considered conservative practice to use a single animal

2 *Care of monkeys* In the proper care of animals the selection of the care-

[illegible]

windows are protected and proper measures have been taken to prevent access to hiding places or the ready escape of the animals into other parts of the laboratory.

a. Caging The placing of many animals (that is, 5 or more) in a large cage is not recommended Opportunities for the spread of disease can be reduced considerably if animals are kept in small numbers (not more than 2, if possible) in smaller cages—a useful size being about 36 inches by 36 inches by 30 inches (see

inoculation depends upon circumstances and objectives The *intracerebral* route is the most reliable, but if the inoculum contains an excess of bacteria, the inoculated monkey may succumb with a brain abscess before it acquires experimental poliomyelitis The *intraspinal route* is delicate and sometimes yields a quicker answer, but the size of the inoculum is necessarily small. The *intranasal* route is less reliable and not dangerous to the animal but is cumbersome and time-consuming The *intra-abdominal* route is also less reliable but is simple and safe and is often used as an adjunct to the other two routes

a *Intracerebral inoculation* The monkey should be anesthetized, the hair may be clipped away from the top of the head, and this area can then be shaved The site of inoculation is the central area over the frontal lobe on the right or left side The skin over this area should be rubbed well with iodine and alcohol Trephining of the skull can be accomplished by using a sharp instrument (half of a pair of scissors is satisfactory), which will bore a hole 1 to 2 mm in diameter, through which 1 ml of the suspension to be tested can be injected intracerebrally It is common practice to inject 1 ml or less, to a depth of about 1 cm.

For fecal material, the intracerebral route of inoculation (alone or in combination with other routes) has been more successful in some hands than in others The danger of the intracerebral method is the ease with which certain fecal suspensions may give rise to a brain abscess in the inoculated monkey

If it is important to try to demonstrate virus by all possible means, the animal may be reinoculated intracerebrally with the same material at intervals of 1, 2, or 3 weeks Cortisone injections have also been used to render the animal more susceptible

b *Intraspinal inoculation* The monkey is anesthetized, and inoculated in the lumbar region of the spinal cord, using a No 20 gauge needle. The inoculum should not be larger than 0 1 to 0 2 ml , thus minimizing the chance of damage to the cord, which might give rise to a traumatic paralysis The incubation period is shorter than by other routes—the median length being about 4 days

c *Intra-abdominal inoculation* This is carried out in conventional fashion The usual dose is 5 to 12 ml

d. *Intranasal instillation* In carrying out intranasal instillation, it is of value, but not essential, to have the monkey partly anesthetized with ether. The 2 ml of inoculum is allowed to drop directly into each nostril from either a pipette or from a fairly large syringe with a blunt needle attached. During this process it is advisable to have the monkey

[illegible]

... ently in good health

[illegible] arrived in this [illegible] to every 50 [illegible] 13.0 grams; [illegible] 4 5 grams; [illegible] riboflavin, [illegible] scorbic [illegible] about [illegible] tency

Each monkey is fed as many biscuits as it will eat per day (generally 2 or 3). Since they do not contain adequate vitamin C, it is important to supplement the diet by the addition on alternate days of 25 mg of ascorbic acid to the drinking water, to which a small amount of sugar is also added As an extra precaution, about *once per week each animal receives some fresh fruit, generally an orange* Raw peanuts are given several times per week also They may be given as a reward to the monkey for jumping out of its cage, around the runway, and back into its cage; monkeys are trained more quickly this way The use of peanuts is also an aid in the diagnosis of experimental poliomyelitis In contrast to monkeys with brain abscesses, those with poliomyelitis, *regardless of severity, almost never lose their* avidity for peanuts

Sick and malnourished animals may be given supplementary diets: bread with milk and bananas, oranges, and carrots If necessary, fluid intake can be maintained by the administration of saline intraperitoneally

d Marking of animals Tattooing numbers on either the shaved head, forehead, or chest (or other areas) is a useful way of labeling monkeys One also performs a service to fellow research workers by using this method, for, in spite of all efforts, used laboratory animals may find their way back to the dealers and will be resold presumably as unused animals unless they are marked with a characteristic tattoo number.

Another common method of labeling a monkey, which is far less satisfactory, is to place around the neck of the animal a collar and chain to which a metal tag is attached Care should be exercised to see that the tag does not become detached and that the chain or collar does not injure the skin of the neck

3. *Routes of inoculation of test monkey* The choice of route of

Even in the absence of signs, it may be wise to sacrifice the animal routinely at the end of the 30-day period of observation. It is not good practice to use the animal over again for poliomyelitis investigation

b. *Autopsy of monkeys* An animal to be sacrificed can be killed by the injection of ether into the heart. The large blood vessels of the neck are cut, and the blood allowed to drain from the monkey. Before incising the skin, the fur of the back and head of the animal should be swabbed down with lysol solution. The brain and cord are removed first, using 2 or 3 changes of sterile instruments It is useful to remove the cord with the dura intact and then to open the dura with sterile scissors and forceps Several sections are taken from 3 levels of this cord (cervical, thoracic, and lumbar) and from the medulla. One of each is placed in fixing solution for eventual histologic study, and several of each are kept for passage or storage, either frozen or in 50 per cent glycerol.

c. *Criteria for a positive result.* The most important evidence of the experimental disease in the monkey lies in the histologic examination of the sections taken from the spinal cord Some animals may have failed to develop signs of experimental infection during life, but lesions of the disease are found in the central nervous system, usually less extensive than in paralyzed monkeys The lesions should be unequivocal before a positive diagnosis of infection with poliomyelitis virus is accepted. Such lesions are generally manifest in the gray matter of the spinal cord, involving in particular the ganglion cells in the anterior horns. The lesions pass through several stages, but at their height, and later, are characterized by destruction of neurons and neuronophagia and, prominently, by perivascular and interstitial round-cell infiltration

In the brain, lesions are not likely to be extensive and may be scattered, often in the base of the brain and with some selectivity for the motor cortex Such cerebral lesions are not convincing evidence of poliomyelitis unless more lesions are also found in the medulla and other levels of the cord By adherence to this rule, one will less readily confuse experimental poliomyelitis with other types of encephalomyelitis or areas of inflammation surrounding a brain abscess or brain injury It should be recalled that occasionally viruses other than poliomyelitis may give rise to a myelitis when inoculated intracerebrally Consequently it may be wise to pass the strain of virus in tissue culture and test its specificity by subjecting it to neutralization with the three types of poliomyelitis antisera, or to pass the strain to another monkey for confirmatory evidence Under certain circumstances, it may also be wise to test the strain intracerebrally in adult and suckling mice, hamsters, cot-

held underneath a fixed glass plate by an assistant, so that splattering of the infectious material can be somewhat controlled. If the glass plate is not available, the operators should wear goggles and cellophane masks. Instillation of material is advised to be carried out daily over a period of 3 to 6 days

4. *Observation of inoculated monkeys.*

a. *Signs of experimental poliomyelitis.* Monkeys inoculated with material suspected of containing poliomyelitis virus should be observed for at least 4 weeks from the time of the last inoculation, and daily records made of the observations (see Fig. 2). It is good practice to examine and exercise the animals daily during this period. By this method, such early signs of poliomyelitis as tremors, ataxia, and weakness of the limbs can be detected, and the animal can promptly be sacrificed at an appropriate time if a strain of virus is desired. The further value of daily examinations is based on the fact that soon after an intracerebral or intraspinal inoculation, the animal may get a spastic paralysis, often manifest as a hemiplegia caused by an upper motor lesion, or a flaccid paralysis, resulting from the local trauma or necrosis of the brain, or the cord, respectively. It is important not to confuse these lesions with flaccid paralysis resulting from a poliomyelitis virus infection.

It is desirable to have temperature readings taken daily, preferably at the same time each day, using individual rectal thermometers which are sterilized in a strong disinfectant (10% formalin), between use. This precaution is recommended since thermometers may become contaminated with poliomyelitis virus. The use of a common unsterilized thermometer in a monkey colony has also led to the transmission of tuberculosis via the rectum. The usual rectal temperature of rhesus monkeys varies from 102.2° to 103.5°, but temperatures up to 104° are not particularly abnormal. The onset of the experimental disease (induced with human strains of virus) may follow an incubation period of from 3 to 25 days. This is usually, but by no means always, heralded by a rise of temperature to 104° to 106°. Fever is maintained from 1 to 6 days, during which the development of other signs may appear quickly or slowly. These consist of ruffled fur, nervousness, tremors (often first noticeable as a fine tremor of the ears), ataxia, and finally weakness, to be followed by definite paralysis most easily detectable in the extremities, but it may involve the face, neck, or back. With the development of considerable paralysis there is usually a precipitous fall in temperature.

Blood specimens should be drawn with sterile precautions as for a blood culture. It is desirable to obtain 15 to 25 ml of fasting blood for each determination * The serum should be drawn off about 2 hours after clotting. If it is not possible to separate the serum at this time, the blood should be placed in the refrigerator at 4° C as soon after collection as possible, and the serum removed within the next 24 hours. The serum should be centrifuged, if necessary, to remove traces of erythrocytes, and should be kept in the refrigerator The addition of a preservative to the serum is not recommended If there is to be a delay of more than a few days before the tests can be performed, the sera should be frozen. A commercial freezer set at —20° C. is adequate, or a solid carbon dioxide freezer may be used. The last-named is particularly convenient for use in the field and for transportation of frozen specimens If there is to be a considerable delay before testing the sera, or if they must be held or shipped at room temperatures, lyophilization may be used.

B The Neutralization Test

This test as carried out with poliomyelitis virus in mice has been supplanted in many laboratories by the use of tissue cultures for the purpose (*see* Chapter III), but the directions for the mouse test have been included for those to whom tissue culture facilities are not available. The use of monkeys in the performance of neutralization tests with poliomyelitis virus is no longer recommended A broad experience has accumulated over the past 15 years with the intracerebral test in mice for Lansing (Type 2) antibodies (section III, B, 2). Recently, with the adaptation of Type 1 and 3 strains to mice, an intraspinal test for Type 1 and 3 antibodies has become available (section III, B, 3).

1 *General principles.* The principles of the neutralization test are that specific protective, or virus-neutralizing, antibodies can be measured by adding serum containing them to virus and then injecting the mixture into a susceptible experimental animal, or preferably into a group of such animals The failure of the animals to develop the disease which is acquired by the control animals receiving virus alone, or virus plus a serum free of the antibody in question, is proof of the presence of neutralizing antibodies In poliomyelitis work, the virus-serum mixture may also be inoculated into tissue culture, and failure of virus to grow is indicative of neutralizing antibodies The level of such antibodies can be determined by using a constant amount of virus and falling dilutions of serum or by employing a constant amount of serum and serial dilutions of virus For clinical diagnosis, one must be able to show a significant rise in antibody titer during the course of the infec-

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* *For certain purposes 0 1 ml samples may be necessary This amount can be taken* by puncture of the fingertips, heel, or ear lobe The 0 1 ml of whole blood is aspirated into a calibrated pipette and transferred to a vial containing 0 1 ml of an anticoagulant (heparin) or 1 ml of balanced salt solution ?

ton rats, rabbits, and guinea pigs If these animals develop encephalomyelitis it can be anticipated that one is either not dealing with poliomyelitis virus or, if adult mice alone are infected, that one is dealing with one of the Type 2 (Lansing) strains of poliomyelitis virus. This possibility can be quickly determined by further tests, including attempts to type the virus.

A *negative result* should be recorded if the animal fails to show lesions in the spinal cord of the type requisite for a positive diagnosis In general, the failure of the inoculated animal to develop any appropriate signs during the period of observation is a fair indication of a negative result, but nonparalytic and apparently nonsymptomatic experimental poliomyelitis may occur in a small percentage of inoculated monkeys

An *incomplete* or *unsatisfactory* result is recorded if the inoculated monkey dies from some cause other than poliomyelitis before the 30-day period of observation is complete. An agent indigenous to monkeys produces at times certain histologic changes, but these are ordinarily not confused with the lesions of poliomyelitis

H. Tissue Culture Methods

Advances in this field have been so extensive in recent years that a separate chapter in this book has been devoted to a description of tissue culture methods and uses. While these are based essentially on work with poliomyelitis, they have been applied to other viruses as well

III SEROLOGIC DIAGNOSTIC PROCEDURES

Antibodies may be determined for a number of reasons, including their use in epidemiologic and clinical diagnosis In acute cases, neutralizing and complement-fixing antibodies may already be present at the time the patient is admitted to the hospital (2 or 3 days after the onset of the major illness) The relationship of antibodies to infection is indicated by their quantitative increase during the succeeding few weeks. Presumably the responses to types other than the one causing the current infection are due to broad antigenic groupings.

A Collection of Blood Specimens for Neutralization and Complement Fixation Tests

For epidemiologic surveys, a single blood sample from each person in the study suffices, for clinical diagnostic purposes 2 or more serial samples of serum are desirable if antibodies are to be adequately tested and evaluated. These should be collected as follows: (*a*) as soon as possible after the onset of illness; (*b*) 3 to 4 weeks after onset, and (*c*) later, if necessary for special study

is multiplied by 0.5 and the product added to that nearest dilution which gave over 50 per cent mortality.

e. The *neutralization index* is the expression of the ratio of the virus control LD_{50} titer over the LD_{50} titer of the serum-virus mixtures in which virus has been added to undiluted serum. The logarithm of the LD_{50} titer of the virus control minus the logarithm of the LD_{50} titer of the serum-virus mixture equals the logarithm of the neutralization index. The antilogarithm of this difference equals the neutralization index. A neutralization index of less than 10 is considered to indicate the absence of antibodies.

f. *Screening tests* for poliomyelitis antibodies employ a constant dose of virus and 1 concentration of serum (either undiluted or preferably a 1:5 dilution).

g. *Quantitative tests* for poliomyelitis antibodies usually employ a constant dose of virus (32 to 100 LD_{50}) and varying dilutions of serum (for example 1:4, 1:16, 1:64, 1:256, and 1:1,024). An example is given in Table 9, on page 48.

h. *Interpretation.* As neutralizing antibodies for many viruses persist for long periods, it is essential to demonstrate a rise in titer in paired sera, in order to establish a causal relationship between the virus and a given illness. Such a positive test in a single specimen may be the result of an earlier and perhaps subclinical infection and is useful for epidemiologic purposes, but not for clinical diagnosis of the acute disease. For diagnostic significance, the increase in neutralization index during convalescence should be at least 100. Because poliomyelitis antibodies are usually already present in acute phase sera, their increase during the course of the disease is more readily demonstrated by using a constant amount of virus and varying dilutions of serum.

2. *The intracerebral Lansing (Type 2) test in mice.* An example of a simple method which has been used by several workers for antibodies to Lansing (Type 2) poliomyelitis virus may be cited. This test is run to see whether the individual from whom the serum has been taken has or has not been previously exposed to and infected by an agent which gives rise to Lansing antibodies. It is in essence a *screening* test, which is adequate for certain epidemiologic purposes.[5, 6]

a. Virus. The stock virus pool, obtained from the brains and spinal cords (CNS) of infected mice, should have an LD_{50} titer of not less than $10^{-3.5}$ by the intracerebral route in mice (at least 3 weeks old) from the stock to be used in carrying out the neutralization tests. A control titration is carried out with each neutralization test.

tion A positive test in a single sample of serum is not of diagnostic value insofar as *acute recent infections are concerned*, for neutralizing antibodies can persist for years and their mere presence may indicate a past infection in a given individual In view of the latter fact, neutralization tests are useful in the field of *serologic epidemiology* where one is interested in knowing with which viral agents a given population has had previous experience Therefore, for epidemiologic work, a more simple "screening" test is advised than that used for clinical diagnosis

Although simple in principle, neutralization tests by their very nature are expensive in time and materials, and at their conclusions may be difficult to interpret, *chiefly because of the variability in the titration end point and the possible* nonspecificity of the neutralization For each viral agent, the technic of performing the test for neutralizing antibodies must be standardized Among variables which must be considered are the selection of the experimental animal or tissue culture, route of inoculation of the virus-serum mixture, age of the test animals; stability of the test virus and reproducibility of the end point, relative heat stability of the specific antibody and of possible interfering substances in serum; use of one concentration of virus and varying dilutions of serum or vice versa (and the relationship between varying concentrations of each), temperature at which the neutralizing mixture of virus and serum is held and the time of incubation of the mixture

The following points deserve emphasis in carrying out neutralization tests for poliomyelitis antibodies·

a. Although it is useful to have virus suspensions of known titer available in the frozen state, it is nevertheless necessary to titrate the virus each time a test is performed

b. A standard *reference serum* of known antibody titer is desirable.

c. *For the mouse test a stock of mice of known uniform susceptibility should be selected, and mice 3 to 4 weeks of age should be used* routinely They are inoculated intracerebrally (or intraspinally) with 0 03 ml of the virus (or virus-serum mixture) They are observed daily for signs of weakness or paralysis, especially of the forelegs, to establish specificity of the deaths All illnesses and deaths are recorded daily for a period of 21 days All deaths within 24 hours after inoculation are regarded as due to traumatic or nonviral causes

d. The 50 per cent end points (ID_{50}, LD_{50}, or TCD_{50}) are calculated according to the method of Reed and Muench,[3] or of Kärber,[4] the titer being expressed in terms of the concentration of infected mouse brain and spinal cord or of tissue culture fluid, see pages 44 to 49.

In the tissue culture calculations, the mortality ratio (no of mice dead/no. of mice inoculated) is replaced by the cytopathogenic ratio (no. of cultures showing cytopathic changes/no of cultures inoculated).

If the successive dilutions of virus are carried out in 0.5 log steps, rather than in the full log steps in the example, the proportionate distance

is multiplied by 0 5 and the product added to that nearest dilution which gave over 50 per cent mortality

e The *neutralization index* is the expression of the ratio of the virus control LD_{50} titer over the LD_{50} titer of the serum-virus mixtures in which virus has been added to undiluted serum The logarithm of the LD_{50} titer of the virus control minus the logarithm of the LD_{50} titer of the serum-virus mixture equals the logarithm of the neutralization index The antilogarithm of this difference equals the neutralization index A neutralization index of less than 10 is considered to indicate the absence of antibodies.

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2 *The intracerebral Lansing (Type 2) test in mice* An example of a simple method which has been used by several workers for antibodies to Lansing (Type 2) poliomyelitis virus may be cited This test is run to see whether the individual from whom the serum has been taken has or has not been previously exposed to and infected by an agent which gives rise to Lansing antibodies It is in essence a *screening* test, which is adequate for certain epidemiologic purposes [5,6]

a Virus The stock virus pool, obtained from the brains and spinal cords (CNS) of infected mice, should have an LD_{50} titer of not less than $10^{-3.5}$ by the intracerebral route in mice (at least 3 weeks old) from the stock to be used in carrying out the neutralization tests A control titration is carried out with each neutralization test.

The pool of infected mouse CNS, in the form of a 20 per cent suspension in distilled water, should be distributed in ampules or in tightly stoppered tubes, frozen and kept at −20° C. or below. Samples are thawed as needed or centrifuged at low speeds and the supernatant fluid diluted to the proper amount (see below)

b. Sera For survey purposes samples of blood should be collected from normal individuals covering as wide an age group as is feasible. If the series is to span all age groups there should be adequate representation of the youngest age groups if possible, that is, 1 month to 5 years, and the whole series should include upwards of 200 samples.

Sera should be handled aseptically and ideally should be stored frozen from the time of collection and thawed just before use. (Sera may be heated at 56° C for 30 minutes, the antibody being stable under these conditions, in order to obviate the possibility that thermolabile nonspecific factors might give false positive tests for antibody.) A standard reference serum is desirable.

c. Procedure From the accumulated values of 4 titrations of the virus pool, using 8 mice per dilution, the extent to which the 20 per cent suspension must be diluted is calculated so that the final virus-serum mixture contains 100 LD_{50} per 0.03 ml, the actual volume inoculated into the brain For example, if the virus pool has an average titer of $10^{-3.5}$ based on the wet weight of the CNS used to prepare the suspension, then the concentration of infected CNS desired in the final mixture is $10^{-1.5}$. The virus suspension which is added to the serum must be twice as concentrated for it will be diluted 2-fold by the serum Therefore, in the present example, virus-infected CNS in a concentration of $10^{-1.2}$ is required

20% suspension $= 10^{-0.7}$ concentration of CNS
Desired concentration $= 10^{-1.2}$
Dilution necessary $= 10^{-0.5}$
Antilog of 0.5 = 3.2

Therefore, the virus suspension to be used in the test is prepared by adding 2.2 ml of distilled water to each ml of 20 per cent suspension

With each series of neutralization tests, control titrations of virus are done in the presence of saline. If the control titration is unsatisfactory, only certain sera must be retested For example, if the control titration should show that only 10 LD_{50} were added to the sera under test, then those sera negative in the test need not be run again, for they will not neutralize larger doses of virus. On the other hand, if the control titration should show that 1,000 LD_{50} were used in the test, the positive sera need not be run again for they will neutralize smaller

doses of virus In deciding whether or not a test should be repeated, a variation of 0 4 log is permissible, that is, control titrations ranging from $10^{-3.1}$ to $10^{-3.9}$ are allowable for a virus which has given an average titer of $10^{-3.5}$ and if the test in question was based on this latter value Thus, although the calculated dose is 100 LD_{50}, tests in which the control titrations show that 40 to 250 LD_{50} were used need not be repeated

To 0 2 ml of serum in a 10 by 75 mm test tube 0 2 ml of the virus suspension are added The mixture is allowed to stand at room temperature (about 20° to 25° C) for 1 hour For each serum to be tested, 8 mice are inoculated intracerebrally with 0 02 ml of serum-virus mixture

Mice are examined once (or twice) daily for 3 weeks, with paralyzed and dead animals being recorded In a small percentage of the tests 1 or 2 of the 8 mice may die during the first 24 hours, and such deaths are not tabulated Under the latter circumstances the results are based on 7 or 6 rather than on 8 mice

d Critical level of antibody The criteria for the "presence" of neutralizing antibody depend upon the number of surviving mice 1 day after the injection and the number surviving 21 days later These criteria are listed in Table 1

TABLE 1

THE EVALUATION OF NEUTRALIZATION TEST RESULTS

No of mice surviving 1 day after inoculation	Number of mice that succumb during observation period of 3 weeks		
	Positive test, antibody present	Negative test, antibody absent	Questionable test*
8	0, 1, 2	6, 7, 8	3, 4, 5
7	0, 1, 2	5, 6, 7	3, 4
6	0, 1	5, 6	2, 3, 4

* Should be repeated, if possible

e. If *quantitative* serum titers are desired, the serum should be diluted to appropriate levels, such as 1 5, 1:25, 1 125, and 1 625

To 0 2 ml of each serum mixture, there is added 0 2 ml of the test virus After incubation as above, 8 mice are inoculated with each serum-virus mixture. Fifty per cent serum end points are calculated according to Reed and Muench or Karber methods

3. *The intraspinal test in mice for Types 1, 2, and 3 antibodies* *

* We are grateful to Dr Morris Schaeffer and Dr C P Li for these directions

Recently Li and Schaeffer[7] have devised a method of performing neutralization tests in mice against the three virus types with mouse-adapted strains. The technic is still new and has not had thorough evaluation. Further experience may suggest additional modifications; however, the general principles are the same as for the Lansing neutralization test in mice, described in the above sections. The intraspinal method is described by them as follows:

Viruses. a. Type I. Mahoney strain, adapted to mice. This virus after the 45th intraspinal passage in mice has an ID_{50} titer of $10^{-3.5}$ to $10^{-4.0}$. A large number of mice (100 – 100) are inoculated with the seed virus, and those mice showing the first signs of paralysis are sacrificed and their cords harvested. Ten per cent suspensions of those cords are made in buffered saline by grinding first in a mortar, then in a tissue grinder (TenBroeck type). The suspensions are centrifuged at 1,500 r.p.m. for 5 to 10 minutes, and the supernate transferred to a flask immersed in an ice bath. The virus suspension is then distributed to 2 ml. ampules with a syringe. One ml. is delivered to each ampule while the flask is shaken periodically during the process of distribution in order to have an even suspension in each ampule. The ampules are sealed and rapidly frozen with CO_2 ice and alcohol and stored in a dry-ice refrigerator at -50° to -70° C. The ampules are removed and used as needed. Random ampules are titrated from time to time to check on the stability of the virus titer.

b. Type II. Any Type II mouse-adapted strain of high titer, such as MEF-1, may be used. After a few intraspinal passages in adult mice, stock cord suspensions diluted to 1:50 and 1:500 are made as described above. This virus has a titer of about $10^{-3.5}$ by the intraspinal route in adult mice.

c. Type III. Leon strain, adapted to mice. The titer of this virus even after 100 passages in mice is only $10^{-2.5}$ to $10^{-3.0}$. Cord suspensions are prepared as described for Type I, except that a heavy dose of the seed virus is still necessary for inoculation of mice in the preparation of the stock virus. A dose of 0.03 ml. of 20 per cent suspension of the seed virus is inoculated into each mouse. With this heavy suspension some of the mice die immediately after inoculation, but those which survive and become paralyzed will yield cord suspensions of maximum titer.

d. Seed virus and working virus. For accurate neutralization tests a large quantity of standard stock virus is desirable; once prepared, it can be stored frozen for a long time. To obtain comparable results in later tests, the virus, when exhausted, should be replaced from the same seed virus if possible. Therefore, sufficient quantity of seed virus should be kept on hand.

e. Sera. Same as for Lansing neutralization test in mice.

f. Mice. Mice 4 to 5 weeks old, weighing 12 to 16 grams are recommended, although older mice may be used.

g. Procedure. The lowest dose of virus to give reproducible results is recommended for the neutralization test. With mice of uniform susceptibility, a dose of 10 ID_{50} has been found to give reliable results without false positives. Doses above 30 ID_{50}, under these circumstances, may be too large, for weakly positive sera may be missed.

Stock virus is diluted appropriately with buffered saline so that when mixed with an equal amount of serum, the mixture will be equivalent to 10 ID_{50} per 0 02 ml. of inoculum The calculated dilution of the virus, in 0 2 ml amounts, is mixed with undiluted inactivated serum, for qualitative screening tests, and with serial dilutions of serum for quantitative antibody tests, as described in the above sections The mixture is incubated at 37° C for 1 hour and then 0 02 ml is inoculated into mice intraspinally. Eight mice are used for each dilution. Immediately after inoculation the mice are examined, and if any deaths occur because of anesthesia or trauma, additional mice are inoculated to make up the stated number

h. Intraspinal technic Under ether anesthesia the middle of the back of the mouse is wetted down with alcohol and a transverse incision through the skin is made with small iris scissors (Fig 3) The mouse is then held in one hand in such a way that the vertebral column is slightly flexed With a ¼-inch 27-gauge, short-beveled needle and a 0 25 ml tuberculin syringe, the needle point is introduced between the vertebrae at the level of the lumbar enlargement of the cord, slightly to the right of the midline (Fig 4) The direction of the pathway of the needle as slight pressure is applied is at about a 45° angle toward the head and at a very slight angle toward the midline As the point of the needle enters the spinal canal a definite "giving" sensation is felt by the hand holding the syringe A volume of 0 02 ml is then injected and the needle removed along the same pathway by which it was introduced No applications or dressings are applied to the skin wound, which heals without difficulty

Once mastered, this technic goes rather rapidly With one assistant anesthetizing the mice, one technician can readily inoculate 100 mice in 1 hour's time The age of mice affects the ease with which intraspinal inoculation may be accomplished Best results have been obtained with 4-week-old mice, but difficulty of introducing the needle between the vertebrae is not encountered unless mice over 6 weeks of age are used Although this is a relatively greater traumatizing procedure compared with the intracerebral technic, the loss of mice should be weighed against the advantages of reduction in incubation period for Type II, and the making of possible determinations of Types I and III antibodies in mice.

i Observation The test mice are examined 24 hours after inoculation, and deaths or traumatic paralysis noted The dead mice are discarded and those affected with traumatic paralysis (about 7%) are marked with dye to differentiate them from the virus-paralyzed mice.

Traumatized mice frequently recover or at least will not become worse unless virus paralysis is superimposed. Traumatic paralysis can be differentiated readily from virus paralysis since virus paralysis does not occur during the first 24 hours. Traumatic paralysis always appears during this period.

The incubation period for Type I and Type II with the dosage indicated (10 ID_{50}) is usually 3 or 4 days and that for Type III is 4 or 5 days An observation period of 7 days is sufficient to complete the test. Positive results are those in which of 8, 7, or 6 mice (alive 24 hours after the inoculation) half or more survive a 7-day observation period; negative tests are those in which fewer than half of the mice survive If half of the animals survive, the test should be repeated.

Occasionally, mice affected with poliomyelitis virus via the intraspinal route die of infection on or after the 3d day of inoculation without observed paralysis This occurs more frequently with Type II virus than with Types I and III.

4. *The neutralization test in tissue culture.* All of the tissue culture methods outlined in Chapter III lend themselves readily to the measurement of poliomyelitis neutralizing antibody, since antibody specifically neutralizes the cytopathogenic action of the virus If a constant dose of virus is used, the 50 per cent neutralizing end point of the serum can be calculated in accordance with the methods outlined above for the mouse neutralization tests. Details of a number of tissue culture procedures may be found in Chapter III

Poliomyelitis laboratories [7,13] differ somewhat in the details of performing this test, but the same principle underlies them all. For purposes of illustration, the test which has seen considerable use in this laboratory will be described. In this test, cultures of monkey kidney epithelial cells grown on glass in a 16 by 150 mm test tube are used.[14,15] Cultures are maintained in a stationary position as outlined in Chapter III. 1 ml. of fluid medium is present in each culture.

a Virus It is essential that representative strains producing sharp, rapid cytopathogenic effects be used, such as Brunhilde for Type 1, Y-SK for Type 2, and Leon for Type 3 Virus is propagated in tissue culture, and a pool of high titer is prepared and centrifuged at low speed to remove cells and debris The virus is distributed *in small aliquots (0 5 ml amounts) and frozen at –20° or below A fresh* [illegible] thogenic [illegible] lculated, [illegible]

b. Virus titration Serial 10-fold dilutions are prepared in balanced salt solution (BSS) to which antibiotics have been added One-tenth ml. of each dilution is introduced into 4 tissue culture tubes, and microscopic readings at 100 × magnification are made 3 days later to determine the 50 per cent cytopathogenic end

point This is calculated by the method of Kärber or that of Reed and Muench, as outlined. With each series of tubes at least 2 cultures are left uninoculated to serve as tissue controls These should show no degeneration during the period of observation, if the test is satisfactory If the virus has a titer of $10^{-5.0}$, then a concentration of 2×10^{-3} will contain 200 TCD_{50} per 0 1 ml, and this may be made as follows

0 5 ml. of undiluted virus plus 2 0 ml. BSS $= 2 \times 10^{-1}$ concentration

0.3 ml of 2×10^{-1} virus plus 29 7 ml BSS $= 2 \times 10^{-3}$ concentration.

c Serum Specimens are collected and stored as described above Before use they are heated at 56° C for 30 minutes, poliomyelitis antibodies are stable under these conditions. Complement or other heat-labile accessory factors which may be present in serum are not required in the neutralization test [11]

d. Procedure Serial dilutions of the heated sera (2-fold or 4-fold increments may be used) are made in BSS containing antibiotics, and 0 2 ml. amounts distributed in 10 by 75 mm test tubes. Two-tenths ml. amounts of virus are added so that the final mixture contains 100 TCD_{50} of virus per 0 1 ml (For special purposes where smaller amounts of antibody are suspected, the final mixture may contain only 10 TCD_{50} of virus. However the test is more reproducible and reliable with the higher dose of virus) The serum-virus tubes are covered with a piece of sterile aluminum foil. After an incubation period of 1 hour at room temperature, 0 1 ml amounts are inoculated into each of 3 tissue culture tubes

With each series of neutralization tests, control titrations of virus are made, the starting concentration being the highest dose (100 TCD_{50})

TABLE 2

TISSUE CULTURE NEUTRALIZATION TEST WITH PAIRED SERA OF PATIENT INFECTED WITH TYPE 1 POLIOMYELITIS VIRUS

Virus*	Serum (day after onset)	Cellular degeneration (cytopathogenic effect) Final serum dilution					50% serum titer	
		1 2	1 10	1 50	1 250	1 1250	Log	Antilog
Type 1	1	0 0 0	+++	+++	+++	+++	0 7	5
	20	0 0 0	0 0 0	0 0 0	0 0 +	+++	2 5	320
Type 2	1	0 0 0	+++	+++	+++	+++	0.7	5
	20	0 0 0	0 0 0	0 + +	+++	+++	1.5	32
Type 3	1	+++	+++	+++	+++	+++	0	0
	20	+++	+++	+++	+++	+++	0	0
None	1	0 0 0						
	20	0 0 0						

* 100 TCD_{50} of each virus used in test.

Traumatized mice frequently recover or at least will not become worse unless virus paralysis is superimposed. Traumatic paralysis can be differentiated readily from virus paralysis since virus paralysis does not occur during the first 24 hours Traumatic paralysis always appears during this period.

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4 *The neutralization test in tissue culture.* All of the tissue culture methods outlined in Chapter III lend themselves readily to the measurement of poliomyelitis neutralizing antibody, since antibody specifically neutralizes the cytopathogenic action of the virus. If a constant dose of virus is used, the 50 per cent neutralizing end point of the serum can be calculated in accordance with the methods outlined above for the mouse neutralization tests. Details of a number of tissue culture procedures may be found in Chapter III.

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b Virus titration Serial 10-fold dilutions are prepared in balanced salt solution (BSS) to which antibiotics have been added One-tenth ml of each dilution is introduced into 4 tissue culture tubes, and microscopic readings at 100 × magnification are made 3 days later to determine the 50 per cent cytopathogenic end

factory for Type 1; Y-SK or MEF-1 for Type 2; and Leon or Saukett [illegible]

and need not generally be treated for anticomplementary activity nor concentrated

Heating the antigens at 56° for 30 minutes destroys all but occasional trace amounts of infectivity and usually any anticomplementary factors As is pointed out under the section on interpretation, the results obtained with heated antigen are not identical to those obtained with unheated antigens Where the laboratory is equipped to handle live virus and a thorough study is desired, serum collected in the acute and convalescent phases should be tested with both heated and unheated antigens At the time of writing no exact substitute for the live virus antigen has been fully tested, but it seems probable that either attenuated strains or formalin-killed virus will prove satisfactory for use in laboratories that are not prepared to handle active, virulent poliovirus

c *Control antigens* Tissue culture fluid from uninfected autolyzed or disintegrated cultures may be used as a control antigen to test for nonspecific reactions with the sera Such reactions are rare

d *Reference sera* Hyperimmune monkey sera prepared against each poliovirus type may be used in the titration of antigens and as positive controls in each test Comparison with standard sera is desirable

1 *Macro (tube) technic* taken from Schmidt and Lennette

a *Diluent* Kolmer saline is used, which contains 0 1 gm of $MgSO_4$ and 8 5 gm of NaCl per liter

b *Complement titration* The complement is titrated in the presence of the specific antigen, and 2 exact units in a volume of 0 2 ml are used in the tests. The incubation period for the titration is 1 hour at 37° C

c *Antigen titration* A box titration of antigen and reference serum dilutions is carried out, using 0 2 ml of antigen undiluted, ½ and ¼ The optimal antigen concentration is that giving a 3 + or 4 + reaction with the highest serum dilution It is desirable to use this concentration of antigens when testing sera for antibodies In practice, it has been found that undiluted fluids may be close to, but slightly below, the optimal level However, these fluids have been used unconcentrated with only slight loss of sensitivity

d *Hemolysin.* 2 units of hemolysin in 0.25 ml, as determined by titration at 37° C for 30 minutes with 1.30 complement (0.2 ml) and a 2 0 per cent sheep cell suspension (0 25 ml) are used

e *Sensitized cells.* Sensitized cells are prepared by mixing equal vol-

used in the serum-virus mixtures. It is also advisable to test the highest concentration of each serum in a tissue culture tube for possible non-specific degeneration. A few tubes are left uninoculated to serve as tissue controls.

The cultures are incubated at 36° C for 3 days and then examined microscopically. At the end of that time the virus titration should show that 100 TCD_{50} had been added to each serum. A typical protocol with matched sera of a patient infected with Type 1 virus is shown in Table 2. A pH color test for neutralizing antibodies which makes use of cell suspensions and which may be carried out in test tubes or disposable plastic panels is described in Chapter III.

C. The Complement Fixation Test *

Infection with poliovirus usually gives rise to complement-fixing (C-F) antibodies which may be detected with antigens from a variety of sources. The first antigens to be used were prepared from infant mouse brains.[16,17] More recently tissue culture fluids have generally been used as an antigen source.[8,18,19,20]

The principle of this reaction is no different from that in any other direct C-F test but many different technics have been used to demonstrate it. Two of these methods will be described here. The macro (tube) method proposed by Schmidt and Lennette[20] can be used in laboratories where various other C-F tests are carried out in test tubes, and under these circumstances the test for poliomyelitis can be added with minimal innovations. The method of Black and Melnick[18] makes use of the Fulton and Dumbell *plate* technic[21] and hence requires smaller quantities of reagents. In this second method, several different concentrations of complement are used with each dilution of antigen or serum in order to determine the amounts of complement fixed and to give added sensitivity by a quantitative estimation of, and compensation for, anticomplementary factors in the reagents. The use of the plate technic does not, however, necessitate the use of multiple levels of complement, and other workers[8,20] have used it for one-dimensional serum titrations similar to those described here for the tube method.

The following reagents are used for both methods:

a. *Complement.* Commercial lyophilized complement may be used.

b. *Antigens.* Virus is grown to high titer ($10^{8.5}$ to 10^{9} TCD_{50} per 0.1 ml.) in bottles of tissue culture (monkey kidney or HeLa cultures are *most commonly* used at present) as described in Chapter III. Any of several strains of poliovirus may be used. Brunhilde or Mahoney is satis-

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* Dr. Francis L. Black and Dr. George L. Le Bouvier assisted in the preparation of this section.

of complement The following control titrations are made with 1, 2, 3, and 4 minimal units of complement:

(1) One and 2 drops of undiluted antigen with hyperimmune sera against the 2 heterotypic poliomyelitis viruses and without sera

(2) The 3 sera at the concentration used in the test without antigen

(3) Complement without antigen or serum

One minimal unit of antigen is defined as the amount required to fix 1 minimal unit of complement in excess of the greatest amount fixed by any of the controls This is between ½ and ¼ of the unit based on fixation of 1 5, 100 per cent lytic units of complement In serum titrations, 8 units of antigen are used in 1 or 2 drops Fluids of a titer lower than 4 units per drop may be concentrated by ultrafiltration or ultracentrifugation

d *Hemolytic system* 0 4 per cent sheep cells are sensitized by incubation for 1 hour at 37° C by an equal amount of 1/200 hemolysin Sensitization carried out in this particular way[21,22] serves to enhance the clumping of unhemolyzed cells, thereby aiding in reading the results

e *Procedure for test* The test is carried out on lucite or perspex sheets (12 by 12 by ¼ inches), the center portions of which are marked off in 100 squares, each measuring 1 inch on a side The squares contain no depressions Materials are placed on the squares by means of dropping pipettes* which deliver 20 cmm per drop These pipettes consist of No 19 hypodermic needles (from which the beveled tips have been cut) fitted onto glass tubes

One plate is set up at a time One drop of the appropriate serum dilution is added to each square on the plate, then 1 or 2 drops of antigen and 1 drop of the appropriate complement concentration The pipette is held 2 to 3 cm above the plate when applying the drops If held lower, the drops may ricochet off those already on the plate, if higher, they spread on the plate and are more subject to evaporation The mixing of reagents which occurs when a new drop is added to one already on the plate is sufficient, no shaking of the plates is required In the controls when one or more reagents are omitted a corresponding number of drops of diluent are added instead

Primary incubation takes place at 4° C for 18 hours To avoid evaporation from the drops during this time, the plates are placed on a rack, which is put in an air-tight box The box always remains in the refrigerator and contains a layer of cotton soaked with water. At the end of the primary incubation, the rack is removed, and any water of condensation forming on the plates is allowed to evaporate before the hemo-

* Macalaster Bicknell Co , Inc , New Haven, Conn

umes of a 2 per cent suspension of sheep erythrocytes and a dilution of hemolysin containing 2 hemolytic units in a volume of 0.25 ml. The mixture is allowed to stand at room temperature for 10 minutes before use.

f. *Procedure for test.* Serial 2-fold dilutions of each serum are prepared in Kolmer saline solution Each dilution is dispensed into 3 series of tubes so that tests against each of the 3 virus types can be run concurrently. In addition, the 1st 2 dilutions of each serum are tested for anticomplementary activity in the absence of antigen and for nonspecific complement-fixing activity with the control antigen Antigen is then added in a volume of 0 2 ml followed by 0 2 ml. of complement dilution containing 2 exact units Fixation is allowed to proceed at 4° C overnight (approximately 18 hours) The tubes are then warmed in a 37° C. water bath for 10 minutes, 0 5 ml of sensitized cells is added to each tube, and secondary incubation at 37° C is then conducted for 15 to 30 minutes, depending upon the time required for the complement control tubes to show clearing The complement controls consist of a series of 4 tubes containing antigen, sensitized red cells, and 2 units, 1½ units, 1 unit, and ½ unit of complement, respectively The tubes containing 1, 1½, and 2 units should show clearing after 15 to 30 minutes of incubation, and as soon as this occurs the tests are read The titer of a serum is the highest dilution (initial, not final) showing 3 + or 4 + fixation with the specific antigen

2. *Plate C-F technic.*

a *Veronal-saline diluent.* A veronal buffer with added Mg^{++} and Ca^{++} is used for making dilutions of all materials in the test. The buffer (pH 7.3) contains in each 100 ml : 0.85 gm. NaCl, 57.5 mg 5,5-diethylbarbituric acid, 37 5 mg sodium-5,5-diethylbarbiturate, 16 8 mg $MgCl_2 \cdot 6H_2O$, and 2 8 mg of $CaCl_2$.

b *Complement titration* A series of about 10 dilutions of complement are made up, ranging from 1/40 to 1/400. One drop of each of these is placed on a plate with 1 drop of a 1/32 dilution of a serum which has previously been found to be free of anticomplementary activity and 1 drop of diluent The serum is necessary to promote clumping of the unhemolyzed cells After the primary incubation the hemolytic system is added, the plate incubated at 37° C., and the degree of hemolysis read One minimal unit of complement is the greatest amount that causes no visible hemolysis This is approximately 1/3 of a 100 per cent lytic unit

c. *Antigen titrations.* Two-fold dilutions of antigen, starting with 2 drops of undiluted TC fluid, and continuing with 1 drop of undiluted, ½, ¼, ⅛, and 1/16 dilutions are titrated with an optimal concentration (¼ to 1/16) of homotypic, hyperimmune monkey serum. The titrations are carried out in vertical columns against 2, 3, 4, and 5 minimal units

as with 1/8 In this example the subtrahend based on the serum control would be 13 for the Type I titration and 8 for Type III, both smaller than those for the antigen controls Since the column with 1 unit of complement appears only in the control, the "avidity" is positive only when the fixation in the test titration is more than 1 complement unit in excess of that fixed in the control This gives the same base line for a significant degree of fixation as is used in determining the serum titer. The avidity appears to be more reproducible and to show fewer erratic variations between serial serum samples than the simple serum titer.

IV INTERPRETATION OF DIAGNOSTIC TESTS CARRIED OUT ON PATIENTS

Acute *paralytic* poliomyelitis is a disease which can usually be diagnosed clinically without too much difficulty But with the *nonparalytic* form of poliomyelitis the reverse is true, laboratory aids are needed more, and actually a considerable percentage of such diagnosed cases may turn out not to be examples of poliovirus infection Nonparalytic poliomyelitis resembles clinically a variety of other infections of the central nervous system, all of which can give rise to such nonspecific symptoms as fever, drowsiness, headache, and stiff neck and can produce an increase of lymphocytes or protein or both in the spinal fluid. Some of the infections simulating nonparalytic poliomyelitis may be differentiated either by using appropriate serologic tests with other antigens (such as mumps virus or lymphocytic choriomeningitis virus) or less commonly by the isolation from the patient of the etiologic agent (such as one of the Coxsackie or ECHO viruses) In a certain percentage of cases of so-called aseptic meningitis or meningoencephalitis, it is not possible, with any test presently available, to say what type of aseptic or meningoencephalitis a particular case may be

A Diagnostic Value of Virus Isolation and Serologic Tests

Of the various diagnostic tests for poliomyelitis now available to the clinician, the *isolation* of poliovirus from the patient is perhaps the most valuable The sources of material from which such isolations can be made include stools and swabs taken from the rectum or throat, of which the stools are most likely to yield the virus [23,25] When the material is collected promptly and sent to the laboratory promptly, the isolation of polioviruses usually can be completed by tissue culture tests in a matter of days so that if an *early* diagnosis is sought, this method has special merit. Virus can be isolated by tissue culture technic from approximately 90 per cent of properly collected stool specimens from early paralytic or serologically confirmed nonparalytic cases [23-25]

lytic system is added It may be helpful to allow an electric fan to play gently upon the plates for 1 or 2 minutes If the day is not humid it may be possible to add the sensitized cells without delay.

Two drops of the sensitized cell mixture are added to each test square The rack is then placed in a 37° C. incubator (or in a box similar to that described above for the primary incubation), which is kept at 75 to 80 per cent saturation by means of water pans. At the end of ½ hour the rack is removed To bring about the clumping of the unlysed red blood cells the plates are either *tapped gently on their edges or allowed to cool* in the refrigerator or both. After completion of the test, the plates may be decontaminated by immersion for at least ½ hour in 2 normal hydrochloric acid or 10 per cent Wescodyne.*

f *Serum titrations* Sera are titrated over the desired range of dilutions with 8 units of each type of poliomyelitis antigen Each titration is carried out with 2, 3, 4, and 5 minimal units of complement. The following control tests are set up, each with 1, 2, 3, and 4 units of complement:

(1) The two highest concentrations of serum used in the titrations without antigen.

(2) The 3 antigens without serum.

(3) Complement without serum or antigen

A diagram of the appearance of a test plate (using only up to 4 units *of complement) and the readings that might be obtained from it are given* in Figures 5 and 6 This serum would have a Type I titer of 16, a Type II titer less than 4, and a Type III titer of 8. As well as the dilution to which a given system will fix 1 unit of complement, it is possible to obtain from such a titration an expression for the amount of complement fixed at any dilution This character of the serum, its avidity for complement, is partially independent of the titer

An expression of the avidity, dependent both on the titer of the serum and on the amount of complement fixed, may be calculated as follows The score for the degree of fixation is summed for all serum dilutions up to the end point of the titration; for example, in Figure 6 the sum for the Type I fixation is 22 and for Type III, 20 From this is subtracted the sum for the degree of fixation in the serum or antigen control, whichever is greater, multiplied by the number of serum dilutions tested up to the end point In this example the subtrahends based on the antigen controls are 3 × 6 for Type I and 2 × 5 for Type III, and the figures for the avidities are 4 and 10, respectively. When the subtrahend was based on the serum control it was usually assumed that there would be the same degree of fixation with 1/16 and 1/32 serum

* West Disinfecting *Company, Long Island City, N.Y.*

gens are more reactive with early sera and make it more difficult to demonstrate an increase in titer. Even with heated antigens, type-specific reactions are often obtained in young children, presumably those with no prior experience with poliovirus, whereas antibodies to 2 or 3 types of poliovirus commonly appear in older age groups

A single *strong* positive reaction may be interpreted as evidence of probable recent experience with poliovirus [26] The C-F antibody response occurring in individuals who have had prior experience with poliomyelitis of any type is usually prompt and often group specific. It is, therefore, often difficult to demonstrate an increase or to identify the type of the current infection in these individuals On the other hand, their early response allows one to make a tentative diagnosis in these cases on the basis of the first serum sample alone This is best achieved with heated antigens

There appear to be two kinds of complement-fixing antibodies formed after infection with poliovirus, particularly in older children and in adults, who presumably have had earlier experience with one or more types of poliovirus·

(1) Antibodies against the group antigen are made rapidly and generally have reached their highest titer by the onset of illness, although in some patients they make their appearance soon after onset and increase in titer over the next few weeks Exposure of Type 1, 2, or 3 poliovirus to heat (or ultraviolet irradiation or formalin) uncovers a previously masked group reactive antigen, common to the three virus types, which is suitable for detecting this group antibody. By using heated antigens, heterotypic fixation (against poliovirus types different from that isolated from the patient) may occur in the absence of corresponding heterotypic neutralizing antibodies

(2) Antibodies against the type-specific antigen associated with the live virus are usually absent in the acute-phase serum and usually appear only after the third week of disease Complement-fixing antibody against the type-specific live antigen is generally not demonstrable in the absence of neutralizing antibody against the same virus type

NOTE—The foregoing procedures have been evolved from experience gained in a number of laboratories including those of the Section of Preventive Medicine Yale University School of Medicine Much of the work in our own laboratories has been aided by grants from the National Foundation for Infantile Paralysis, Inc

IV. REFERENCES

1 Melnick, J L The Ultracentrifuge As an Aid in the Detection of Poliomyelitis Virus *J Exper Med*, 77 195–204, 1943
2 Kokko, U P., Hollander, D H and Turner, T B A Mouse Neutralization Test for Lansing Poliomyelitis Antibody Employing a Small Amount of Whole Blood *Am J Hyg*., 58 377–384, 1953.

The antibody tests above described are also *becoming of increasing* value. For their interpretation in clinical diagnosis, the principle of demonstrating a 4-fold or better rise in homotypic antibodies between the patient's acute and convalescent serum is one to be sought both in neutralization and complement fixation tests. In many instances, however, the patient already has a maximum response in either or both neutralizing and complement-fixing antibodies by the time poliomyelitis is suspected or by the time he or she is admitted to the hospital. Furthermore, it is recognized that infection with one type of poliovirus can induce a transient heterotypic response (most common with complement-fixing) to the other types Heterologous poliovirus antibody responses are rarely elicited by anything but infection with poliovirus and are, therefore, not a major source of error in diagnosis of poliomyelitis per se. The clinician is not so much concerned with a type-specific response of significant titer (for assistance in making a diagnosis of poliomyelitis) as with an antibody response to *any* of the three types of poliovirus. In one series[23] 4-fold neutralizing titer increases were found in 72 per cent of the cases and C-F increases in 49 per cent There are obviously a number of different combinations of rising and falling titers which can be found in the comparison of acute and convalescent sera as tested with neutralizing and complement-fixing antibodies. The percentage of increases demonstrable will vary with the times of serum collection. It should be recalled here that neutralizing antibody titers against poliovirus may decline over a period of years, although seldom reaching insignificant levels, whereas the complement-fixing antibodies may disappear completely within a period of 4 to 20 months.

B. Interpretation When Only C-F Results Are Available

The C-F test may be carried out without test animals, elaborate *equipment, or special safety precautions and hence certain laboratories* may find it possible to use this test when isolation and neutralization tests are not feasible. Two different positive results may be obtained with the C-F test, one, the demonstration of a 4-fold increase in titer with paired sera, the other the detection of a strong reaction (e g total *avidity 15 or greater*[25,26] or titer *of* 32 or *greater in a* line test[20]) in a [illegible] found in approximately [illegible] a [illegible] e [illegible] can be demonstrated.

In order to *demonstrate* an *increase* in C-F antibody titer in a maximum number of cases, specimens (collected as described in section III, A of this chapter) should be tested with unheated antigen. *Heated anti-*

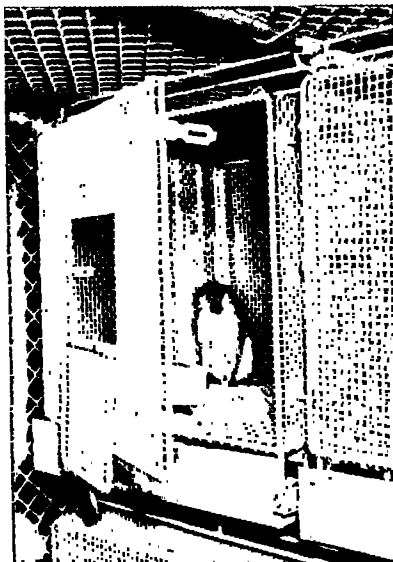


Fig. 1. A convenient-sized monkey cage designed to hold one or two rhesus monkeys. Dimensions of cage: 36 inches by 36 inches by 30 inches fitted below with a sliding tray. The animal in this cage is a green African monkey (*Cercopithecus aethiops sabaeus*).

3. A Simple Method of Estimating Fifty Per Cent End

4. ogischer Reihenversuche.

5. , and Winsor, C. P. Age bodies to Lansing Polio-

6. Pa Antibody

Hyg., 56 151–151,

7. C P and Schaeffer, M Serum Neutralization Tests in Mice and

Virus J Immunol, 72.123–130, 1954.

8. omplement Fixation with the Three Tissue Culture. Am. J. Hyg, 57: 60–70, 1953

9. Salk, J E, Youngner, J S, and Ward, E N. Use of Color Change of Phenol Red as the Indicator in Titrating Poliomyelitis Virus or Its Antibody in a Tissue Culture System Am J Hyg, 60 214–230, 1954

10 Enders, J F The Application of Tissue Cultures to the Preparation and Assay of Viral Antibodies Ann N Y Acad Sci, 58 1072–1084, 1954.

11. Ledinko, N., and Melnick, J L Poliomyelitis Viruses in Tissue Culture. V. Reaction of Virus and Antibody, Variables of the Quantitative Neutralization Test. Am. J. Hyg 59 223–247, 1953

12 Sy P M Studies on the Propagation in

13 I m

14 of
and Med,

15 Method for
–166, 1956

16 Significance
Exper. Med,
96 35–53, 1952

17 Goldblum, N and Melnick, J. L Complement-fixing Antibodies to Type 2 (Lansing) Poliomyelitis Virus in a Normal Population of a Subtropical Area. J Exper. Med, 96 175–185, 1952

18. Black, F L, and Melnick, J L The Specificity of the Complement Fixation Test in Poliomyelitis Yale J Biol. and Med, 26 385–393, 1954

19 Schmidt N J and Lennette, E H A Complement Fixation Test for Poliomyelitis

20 ultraviolet

21 fuenza

22 iruses

23. us and Aseptic Meningitis J A M A,

24 Kibrick, S, Enders, J F, and Robbins, F. C. An Evaluation of the Tissue Culture for the Isolation of Poliomyelitis Viruses from Feces J. Immunol, 75 391–400, 1955

25 Nolan, J P, Wilmer, B H, and Melnick, J. L Poliomyelitis Its Highly Invasive Nature and Narrow Stream of Infection in a Community of High Socioeconomic Level New England J Med, 253.945–954, 1955

26 Melnick, J L, McCarroll, J R, and Horstmann, D M. A Winter Outbreak of Poliomyelitis in New York City The Complement-fixation Test as an Aid in Rapid Diagnosis Am J Hyg., 63 95–114, 1956.

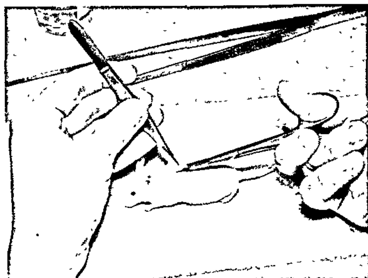


FIG 3 Intraspinal inoculation of mice

Step 1 Preparation of anesthetized mouse for inoculation by clipping the skin in the middle of the back

Photograph reproduced through the kindness of Dr Morris Schaeffer and Dr C P Li U S Public Health Service Communicable Disease Center Virus and Rickettsia Section, Montgomery Alabama



FIG 4 Intraspinal inoculation of mice

Step 2 Injection into spinal canal

Photograph reproduced through the kindness of Dr Morris Schaeffer and Dr C P Li U S Public Health Service, Communicable Disease Center, Virus and Rickettsia Section Montgomery, Alabama

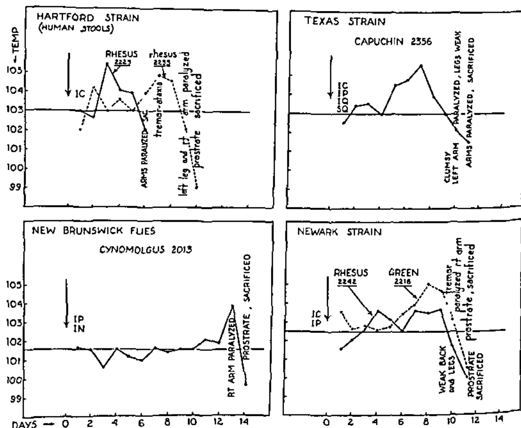


FIG 2 A series of temperature charts that indicate the course of 6 examples of experimental poliomyelitis induced by various routes with 4 different strains of virus in 4 species of monkeys The vertical arrows indicate the time of inoculation, and the routes are designated as IC=intracerebral; IP=intra-abdominal, IQ and SQ=intra- and subcutaneous, and IN=intranasal

In the upper left and lower right corners, the charts show two cases of experimental poliomyelitis in monkeys inoculated simultaneously with the same dose

ECHO VIRUSES*

I INTRODUCTION

With the increased application of tissue culture methods to the diagnosis of poliomyelitis or poliomyelitis-like illness, another group of viruses of wide distribution has been recognized They have been isolated primarily from fecal specimens obtained from patients presenting the clinical syndrome of *aseptic meningitis*, not infrequently called nonparalytic poliomyelitis They have been obtained also from feces of apparently healthy children in the course of epidemiologic studies of poliomyelitis They have been recovered in sporadic fashion but also in association with epidemic prevalences of significant size occurring during the poliomyelitis season. This group of viruses is distinct from the accepted poliomyelitis viruses and from the Coxsackie viruses The designation of echo viruses, enteric cytopathogenic human orphan viruses, *has been generally adopted on the recommendation of a special Committee* on the ECHO Viruses acting under the auspices of the National Foundation for Infantile Paralysis [1]

II CLINICAL AND EPIDEMIOLOGIC FEATURES

A prominent opportunity to obtain uniform information of the clinical manifestations associated with infection by one type of echo virus was presented during the Poliomyelitis Vaccine Evaluation Program of 1954 [2] During that season with the peak in August and early September, 44 cases were reported as possible poliomyelitis from the study areas of Massachusetts and 60 from study areas of central New York state. From a minority of cases poliomyelitis virus was recovered, but from the great majority an "orphan" virus was reported It seems certain from serologic studies that in some patients a double infection occurred Clinically these illnesses presented fever, headache, stiff neck and back, and vomiting, without significant sensory or encephalitic disturbances. Complaint of sore throat and muscle pains occurred in about one-third of the cases Diarrhea was infrequent. There was pleocytosis,

* Editorial addition

	SERUM DILUTION	UNITS OF COMPLEMENT 1	2	3	4	1	2	3	4	SERUM DILUTION	
TYPE I ANTIGEN	1:4		⊙	⊙	⊙		⊙	⊙	⊙	1:4	TYPE III ANTIGEN
	1:8		⊙	⊙	⊙		⊙	⊙	○	1:8	
	1:16		⊙	⊙	○		⊙	○	○	1:16	
	1:32		⊙	○	○		○	○	○	1:32	
TYPE II ANTIGEN	1:4		○	○	○						
	1:8		○	○	○	⊙	⊙	○		TYPE I	ANTIGEN CONTROLS NO SERUM
	1:16		○	○	○	⊙	○	○		TYPE II	
	1:32		⊙	○	○	⊙	○	○		TYPE III	
SERUM CONTROL NO ANTIGEN	1:4	⊙	○	○		⊙	⊙	○		COMPLEMENT CONTROL	
	1:8	⊙	○	○							

Fig 5 Schematic diagram of plate complement fixation test (see text)

	SERUM DILUTION	UNITS OF COMPLEMENT 1	2	3	4	1	2	3	4	SERUM DILUTION	
TYPE I ANTIGEN	1:4		4	3	2		4	4	4	1:4	TYPE III ANTIGEN
	1:8		4	2	1		4	3	1	1:8	
	1:16		4	2	±		2	0	0	1:16	
	1:32		3	0	0		1	0	0	1:32	
TYPE II ANTIGEN	1:4		0	0	0						
	1:8		±	0	0	4	2	0		TYPE I	ANTIGEN CONTROLS NO SERUM
	1:16		±	0	0	3	1	0		TYPE II	
	1:32		1	0	0	4	1	0		TYPE III	
SERUM CONTROL NO ANTIGEN	1:4	3	0	0		4	2	0		COMPLEMENT CONTROL	
	1:8	4	1	0							

Fig 6 Readings of complement fixation of plate shown in Figure 5 (see text)

referred to in previous literature as "orphan viruses"[1] and others as "human enteric viruses"[2]—are now classified as the "enteric cytopathogenic human orphan (echo) group"[5] They share the following properties (i) They are cytopathogenic for monkey and human cells in culture [1-4] All 13 prototype strains were isolated in cultures of monkey kidney cells, which for the strains tested proved to be more susceptible than HeLa cells (ii) They are not neutralized by pools of the three types of poliomyelitis antiserum (iii) They are not neutralized by antiserums for Coxsackie viruses that are known to be cytopathogenic in tissue culture, and they fail to induce disease in infant mice (Animals less than 24 hours old should be used, for they have greater susceptibility) (iv) They are not related to other groups of viruses recoverable

TABLE 1

List of Antigenically Distinct Echo Viruses

Type	Prototype Strain	Geographic Origin*	Illness in Person Yielding Virus	Reference
1	Farouk	Egypt	None	1
2	Cornelis	Connecticut	Aseptic meningitis	1
3	Morrisey	Connecticut	Aseptic meningitis	1
4	Pesascek	Connecticut	Aseptic meningitis	1
5	Noyce	Maine	Aspetic meningitis	1
6	D'Amori	Rhode Island	Aseptic meningitis	1
7	Wallace	Ohio	None†	2
8	Bryson	Ohio	None	2
9	Hill	Ohio	None†	2
10	Lang	Ohio	None	2
11	Gregory	Ohio	None	2
12	Travis 2-85	Philippine Islands	None	3
13	Hamphill 2-188	Philippine Islands	None	3
14	(new type) Tow	Rhode Island	Aspetic meningitis	1

from the alimentary tract (throat or intestine) by inoculation of primate tissue culture, such as herpes simplex, influenza, mumps, measles, varicella, and the ARD (acute respiratory disease) or APC (adenoidal pharyngeal-conjunctival) group (v) They are neutralized by human gamma globulin and by individual human serums, this indicates that they infect human beings

Other studies of the echo viruses (more extensive for some than for others) have provided additional information Complement-fixing antigens have been detected in the culture fluids of a number of viruses that have been tested [1,3] All the viruses tested were ether-resistant Ultrafiltration (gradocol membrane) measurements indicated sizes for types 1, 2, and 3 between 11 and 17 mμ[1] The size of type

comparable to that of poliomyelitis, in the spinal fluid. Two-thirds had less than 150 cells and only 7 per cent more than 500 cells Of the total about 85 per cent were reported nonparalytic, but physical therapists' examinations recorded a mild degree of muscular impairment in the early stages of nearly half the cases A few patients had distinct paralysis and residual disability; there may well have been cases of poliomyelitis, which was also prevalent, in which laboratory identification was not established It was noted that secondary and multiple cases in families were common.

The virus recovered from this epidemic was identified by Kibrick and Enders[3] as type 6 (see below) In 1955 similar outbreaks in eastern and western New York state have also been associated with type 6.[4a,4b] In other areas type 6 has been the echo virus most commonly recovered from cases of this character, but in 1951 type 12 was recovered from a summer outbreak in Massachusetts.[3] Virus has been occasionally recovered from the spinal fluid of patients.[4b]

III ISOLATION AND IDENTIFICATION OF ECHO VIRUSES

The procedures employed for isolation and identification of echo viruses are essentially identical with those for poliomyelitis virus The chapters on poliomyelitis and tissue culture present these details and certain differential features It must be constantly remembered that recovery of an echo virus even with positive serology does not of itself establish the etiologic relation to a sickness. Mixed infections may be common

The further summary of information for identification and classification is taken from the report, December, 1955, of the Committee on the Echo Viruses [1,5]

Preliminary studies of these viruses indicated that multiple antigenic types exist [1,4] Individual prototype strains and serums were exchanged among members of the Committee on the ECHO Viruses for performance of cross-neutralization [illegible] adopted for these tests, employing for the inoculum

[illegible]

cent neutralization of 100 TCD_{50} of virus

This cooperative study has resulted in the differentiation of the 13 antigenically distinct viruses that are listed in Table 1 * These viruses—some of which have been

---

* Another type has been added (ED)

The sera in Pool D are kept separate since they have low titers and must be used in low dilution in the conventional technic. If the plaque neutralization test is used, high serum titers can be demonstrated.

If neutralization occurs with any of the 4 pools, the virus is tested against each individual member of that pool, again using 100 TCD_{50} of virus against a dilution of antiserum containing 20 units of antibody. Caution must be exercised in these procedures for certain animal sera may contain inhibitory substances, perhaps true antibodies against the echo viruses. Thus the new virus might be neutralized, not by antibody produced as a result of the investigator's inoculation of the animal, but by pre-existing antibody naturally present but unknown to the investigator. This difficulty may be overcome by using serum at dilutions sufficiently high to eliminate nonspecific reactions or by testing preimmunization as well as postimmunization serum to demonstrate the lack of pre-existing antibody.

The following table from Dr. Melnick illustrates the differentiating use of neutralization tests.

TABLE 5

RESULTS WITH NEUTRALIZATION TESTS ESTABLISHING TOW STRAIN AS ECHO VIRUS TYPE 14

Antisera	Serum Dilution	Neutralization against 100 TCD_{50} of Tow Virus
Echo Types 1, 2, 3	1:5	0
Coxsackie Pool I*	1:5	0
II*†	1:5	0
A9	1:10	0
B1, B2, B3, B4, B5	1:30	0
Echo Types 1-13 (Tests with sera for each of individual Echo Types 1-13)	1:50	0
Tow, monkey, Rh 8076, preinoc	1:10	0
Rh 8076, postinoc	1:10	+
Rh 8076, " "	1:100	+
Rh 8076, " "	1:1,000	0
Tow, patient, 4th day after onset	1:10	0
93d day after onset	1:10	+
93d day after onset	1:100	+

* Coxsackie serum Pool I: antisera against Types A1, A2, A3, A4, A5, A8, A10, B1, B2, B3.

† Coxsackie serum Pool II: antisera against Types A6, A7, A9, A12, A14.

Monkeys (1 rhesus, 1 cynomolgus) inoculated intracerebrally, intraspinally, and intraperitoneally with Tow strain: asymptomatic. CNS histology negative.

Suckling mice inoculated subcutaneously: asymptomatic.

Complement fixation tests versus specific antigens of other viruses or groups may be done and additional tests with the patient's serum, if available, can be made.

It is apparent that a serologic test of group specificity would be of

10 is reported to be between 60 and 90 mμ[2] Plaque morphology of the echo viruses studied (types 1, 3, 4, 5, 6, 7, and 9) is sufficiently distinctive, except for type 7 (Garnett strain), to permit differentiation from polio virus plaques[6] The plaques of the echo viruses mentioned had irregular diffuse boundaries, and healthy cells could be found within the degenerated areas.

Kidney cells of different monkey species vary in their susceptibility to the echo viruses Rhesus (*Macaca mulatta*) and cynomolgus (*M irus*) cells are susceptible to all 13 types studied Cells from the South American capuchin (*Cebus capucina*) were found to be resistant to types 1, 2, 3, 7, 8, 9, and 11.[2,6] However, they were susceptible to type 10[2] Cells from the African red grass military monkey (*Erythrocebus patas*), which were resistant to types 1, 2, 3, 4, 5, 6, and 9, were as susceptible as those from the rhesus monkey to the type 7 Garnett strain[6]

If requested by other investigators, the committee is prepared to assign numbers to new prototype strains that satisfy the criteria employed for differentiation of the strains listed in Table 1 To avoid unnecessary confusion in the literature, the committee is willing to function as a clearinghouse for characterization of new strains by comparison with established prototypes In this way the distinction of new prototypes may be hastened

If and when any one of the established types is identified as the etiologic agent of a clinically distinct disease, it will be removed from the echo group of viruses

The following details were provided by Dr J L Melnick.

The 50 per cent end point of the virus is calculated from the previous titration and a dilution estimated to contain 100 TCD_{50} is tested in tube cultures against 4 pools of echo antisera prepared in monkeys A parallel virus titration extending 3 to 4 logs beyond the inoculating dose is also performed to establish the correct virus dosage The pools contain antisera to the 14 known antigenically distinct echo viruses and the dilutions are adjusted so that the final concentrations used for neutralization contain, if possible, at least 20 units of each antibody type The composition of the antiserum pools currently in use is shown in Table 2

TABLE 2

Echo Antiserum Typing Pools

Pool A	Pool B	Pool C	Pool D*
Type 2 3 5 6	Type 7 8 9 10 11	Type 1 12 13	Type 4 14
Origin	*Origin*	*Origin*	*Origin*
N. Eng	Ohio	India Egypt Phil Is	N Eng

* Low titers by CPE method, high titers by plaque-reduction method

The sera in Pool D are kept separate since they have low titers and must be used in low dilution in the conventional technic If the plaque neutralization test is used, high serum titers can be demonstrated

If neutralization occurs with any of the 4 pools, the virus is tested against each individual member of that pool, again using 100 TCD_{50} of virus against a dilution of antiserum containing 20 units of antibody Caution must be exercised in these procedures for certain animal sera may contain inhibitory substances, perhaps true antibodies against the echo viruses Thus the new virus might be neutralized, not by antibody produced as a result of the investigator's inoculation of the animal, but by pre-existing antibody naturally present but unknown to the investigator This difficulty may be overcome by using serum at dilutions sufficiently high to eliminate nonspecific reactions or by testing preimmunization as well as postimmunization serum to demonstrate the lack of pre-existing antibody

The following table from Dr Melnick illustrates the differentiating use of neutralization tests

TABLE 3

Results with Neutralization Tests Establishing Tow Strain as Echo Virus Type 14

Antisera	Serum Dilution	Neutralization against 100 TCD_{50} of Tow Virus
Polio Types 1, 2, 3	1/5	0
Coxsackie Pool I*	1/5	0
II*†	1/3	0
A9	1/10	0
B1, B2, B3, B4, B5	1/30	0
Echo Types 1-13 (Tests with sera for each of individual Echo Types 1-13)	1/50	0
Tow, monkey, Rh 8076, preinoc	1/10	0
Rh 8076, postinoc	1/10	+
Rh 8076, " "	1/100	+
Rh 8076, " "	1/1,000	0
Tow, patient, 4th day after onset	1/10	0
93d day after onset	1/10	+
93d day after onset	1/100	+

* Coxsackie serum Pool I antisera against Types A1, A2, A3 A4, A5, A8, A10, B1, B2, B3

† Coxsackie serum Pool II antisera against Types A6, A7, A9, A12, A14

Monkeys (1 rhesus 1 cynomolgus) inoculated intracerebrally, intraspinally, and intraperitoneally with Tow strain asymptomatic CNS histology negative

Suckling mice inoculated subcutaneously asymptomatic

Complement fixation tests versus specific antigens of other viruses or groups may be done and additional tests with the patient's serum, if available, can be made

It is apparent that a serologic test of group specificity would be of

10 is reported to be between 60 and 90 mμ [2] Plaque morphology of the echo viruses studied (types 1, 3, 4, 5, 6, 7, and 9) is sufficiently distinctive, except for type 7 (Garnett strain), to permit differentiation from polio virus plaques [6] The plaques of the echo viruses mentioned had irregular diffuse boundaries, and healthy cells could be found within the degenerated areas

Kidney cells of different monkey species vary in their susceptibility to the echo viruses Rhesus (*Macaca mulatta*) and cynomolgus (*M irus*) cells are susceptible to all 13 types studied Cells from the South American capuchin (*Cebus capucina*) were found to be resistant to types 1, 2, 3, 7, 8, 9, and 11.[2,6] However, they were susceptible to type 10.[2] Cells from the African red grass military monkey (*Erythrocebus patas*), which were resistant to types 1, 2, 3, 4, 5, 6, and 9, were as susceptible as those from the rhesus monkey to the type 7 Garnett strain [6]

If requested by other investigators, the committee is prepared to assign numbers to new prototype strains that satisfy the criteria employed for differentiation of the strains listed in Table 1 To avoid unnecessary confusion in the literature, the committee is willing to function as a clearinghouse for characterization of new strains by comparison with established prototypes In this way the distinction of new prototypes may be hastened

If and when any one of the established types is identified as the etiologic agent of a clinically distinct disease, it will be removed from the echo group of viruses

The following details were provided by Dr J. L. Melnick.

The 50 per cent end point of the virus is calculated from the previous titration and a dilution estimated to contain 100 TCD_{50} is tested in tube cultures against 4 pools of echo antisera prepared in monkeys A parallel virus titration extending 3 to 4 logs beyond the inoculating dose is also performed to establish the correct virus dosage The pools contain antisera to the 14 known antigenically distinct echo viruses and the dilutions are adjusted so that the final concentrations used for neutralization contain, if possible, at least 20 units of each antibody type The composition of the antiserum pools currently in use is shown in Table 2

TABLE 2

ECHO ANTISERUM TYPING POOLS

Pool A	Pool B	Pool C	Pool D*
Type 2 3 5 6	Type 7 8 9 10 11	Type 1 12 13	Type 4 14
Origin	*Origin*	*Origin*	*Origin*
N Eng	Ohio	India Egypt Phil Is	N Eng

* Low titers by CPE method, high titers by plaque-reduction method

TISSUE CULTURE METHODS FOR THE CULTIVATION OF POLIOMYELITIS AND OTHER VIRUSES*

JOSEPH L. MELNICK

Professor of Epidemiology,
Yale University School of Medicine,
New Haven, Connecticut

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* The methods described have been developed in a number of laboratories, chiefly with the [illegible]

great advantage in diagnostic work Studies with complement-fixation tests are not yet sufficiently developed to know whether in this group as with the adenoviruses (RI-APC) a common identifying component can be demonstrated serologically with patients' sera.

A Conference on Viruses in Search of Disease was held by the Section of Biology of the New York Academy of Sciences on May 24 to 25, 1956 Publication of the conference will provide additional information of the distribution of the echo group.

IV. REFERENCES

1 Committee on ECHO Viruses, National Foundation for Infantile Paralysis, Dalldorf, G, Enders J F, Hammon, W McD, Sabin, A B, Syverton, J T., and Melnick, J. L "Enteric Cytopathogenic Human Orphan (ECHO) Viruses." *Science,* 122 1187–88, 1955

2 Poliomyelitis Vaccine Evaluation Center Francis, T, Jr, *et al* "Evaluation of the 1954 Poliomyelitis Vaccine Trials" *Am J Pub Health,* 45 May Supplement, 1955

3 Kibrick, S and Enders, J F In press

4a Dalldorf, G Personal communication

4b Karzon, D T Personal communication

5. References from ECHO Committee Report

 1 J L Melnick, *Am J Public Health* 44, 571 (1954) *Ann N Y Acad Sci* 61, 754 (1955), unpublished data obtained with A D Macrae, E. C Curnen, J T Riordan, J Emmons, S Melnick, D Banker, and P N Bhatt

 2 M Ramos-Alvarez and A B Sabin, *Proc Soc Exptl Biol Med* 87 655 (1954), *Am J Public Health,* in press

 3 W McD Hammon and E H Ludwig, unpublished data

 4 J F Enders, unpublished data

 5 At a moment of conviviality, F Duran Reynals, Yale University School of Medicine, first suggested that viruses in search of disease be called orphan viruses

 6 G D Hsiung and J L Melnick, *Virology,* in press; unpublished data

tories,[1] so that in the present stage of rapid progress and frequently changing technics, a standard procedure cannot be recommended The choice of methods is often dictated by the local availability of suitable tissues and other materials, therefore, certain selected methods that have given satisfactory results in different laboratories will be reviewed

All viruses isolated in tissue culture from patients suspected of having poliomyelitis do not belong in the poliomyelitis family.[1] Some Coxsackie viruses, especially those belonging to Type A-9 and all five B types, are cytopathogenic in tissue cultures of human or monkey cells Some of the fecal specimens that yield these agents may be negative in mice, and it is only when infected tissue culture fluids are tested in infant mice that the virus may be recognized A number of other agents have been isolated, chiefly from feces of patients with aseptic (or lymphocytic) meningitis, and from normal children Some of the new agents ("orphan" or "echo" viruses) are the same size as poliomyelitis virus and have certain other properties in common.[3,54] They are not pathogenic for monkeys or mice and are antigenically distinct from the poliomyelitis and Coxsackie viruses

Another group of agents that have been recovered from the nasopharynx and from feces must also be considered These have been isolated in tissue culture from patients with acute respiratory disease,[4] and related agents have also been isolated in tissue culture from a high percentage of adenoids of children [5]

A General Methods

Poliomyelitis virus has been cultivated by two main methods, *(a) suspended-cell* cultures in flasks and *(b)* tube cultures either *rolled* or held *stationary* and readily examined for cytopathic changes under the microscope Tube cultures are recommended for most work, in view of the rapidity and ease with which results are obtained The plaque technic of Dulbecco and Vogt[26] offers great advantages for precise measurements of virus and antibody Cell suspensions are recommended for routine neutralization tests [37–41]

Poliomyelitis virus has been cultivated in the following tissues[1]. *human embryonic* skin, muscle, intestine, kidney, and lung, *human postnatal* kidney, testis, uterus, prepuce, tonsil, and subcutaneous tissue, *monkey* testis, kidney, lung, and muscle In addition, cultured strains of cells derived from human cancerous tissue have been used [6,7] It is

I INTRODUCTION

The discovery reported in *1949* by *Enders, Weller, and Robbins*[2] that poliomyelitis viruses can multiply in vitro in cultures of human tissue has led to a revolution in methods in the virus laboratory. The methods of tissue culture have proved to be faster, more convenient, and more accurate than methods necessitating the inoculation of monkeys or mice Technics have been developed for the isolation and antigenic [illegible] materials, quantita- [illegible] made in tissue cul- [illegible] pared from infected cultures Different technical methods are now used in different labora-

sterile towel, and kept at 5° C until dissected, usually dissection is carried out 1 to 3 hours after hysterotomy The skin, muscle, and connective tissue, customarily referred to as "skin-muscle," is the material often used, mostly dissected from the arms and legs The lung, too, is particularly useful, and other tissue may also be taken

2 *Human postnatal tissues* Preputial tissues may be obtained from individuals ranging in age from 3 months to 11 years, in whom circumcision has been performed by the dorsal slit method after preparation of the skin with tincture of green soap and zephiran The prepuce is covered with sterile moist gauze and kept at 5° C until it is ready to be minced, a procedure usually carried out within 4 hours of removal The ring of tissue is opened, washed in 3 changes of medium, each consisting of about 15 ml., and then minced When tissues, including foreskin, are known to be contaminated with bacteria, the antibiotic content of the medium used for storage and that first added to the cultures should be at least 100 units of penicillin and 100 μg of streptomycin per ml

Normal *kidney* tissue from infants ranging in age from 1 to 9 months is obtained as a by-product of the operation of ureteroanastomosis Portions of the kidney cortex and medulla are minced within a few hours of removal Since the resulting tissue suspension is usually very turbid, the minced tissue is washed 3 or 4 times with several volumes of medium Occasionally, satisfactory kidney tissue can be obtained at autopsy from infants aged from 1 week to 7 months Other types of human tissue obtained at operation may be used These include tonsils, thyroid, testis, and uterus

3 *Monkey tissues* A variety of monkey tissues, particularly kidney and testicle, have also been found to support the growth of poliomyelitis virus

4 *Mincing of tissues* Tissues are cut into large pieces (about 1 g) and transferred in amounts not exceeding 10 g to a 50 ml round-bottom centrifuge tube for mincing A small quantity of balanced salt solution is added to prevent desiccation of the tissues during mincing This is accomplished by repeated cutting with long-handled scissors until the pieces are reduced to approximately 1 to 2 mm in diameter A volume of medium equivalent to that of the minced tissue is then added Cultures are usually prepared as soon as mincing is completed The minced tissue can, however, be preserved for varying periods as described later.

5 *Storage of tissue* After mincing with scissors (or passing through a Fischer press), fragments are placed in 250 ml flasks containing 20 ml medium Sufficient tissue is added to cover the bottom of the flask with a single layer of fragments The flasks are tightly fitted with rubber stoppers and kept at about 5° C Virus may be propagated with tissues kept for periods as long as 19 days under these conditions

It has also been found feasible to store tissues in the form of finished cultures Suspended-cell cultures of embryonic skin-muscle prepared with medium containing 0.2 per cent human albumin and stored at 5° C
quently supported the multiplication of virus

difficult to recommend one of these tissues in preference to another, and final choice must depend upon their local availability.

Several different media have been used. Most contain a balanced and buffered salt solution with the addition of such animal materials as horse or bovine serum, bovine serum ultrafiltrate, bovine or chick embryo extract, beef amniotic fluid, and hydrolysates of nutritionally complete proteins such as lactalbumin (see Appendix). Synthetic nutrient media[8,30] have been found desirable for many procedures. It is customary to add antibiotics to all media to facilitate the maintenance of bacterial sterility Weller, Enders *et al.*[9] recommend that all manipulations be performed in a chamber or "hood" Before use, the hood is wiped out, a towel wet with lysol is placed on the work surface, and the interior of the chamber is "steamed" for about 10 minutes with the door closed Mycotic or bacterial contaminants have been encountered only rarely when this simple procedure has been followed, and antibiotics included in the medium

II. SUSPENDED-CELL CULTURES

This method is an application of the Maitland-type tissue culture to poliomyelitis and is described in detail by Weller *et al* [9] Cell growth does not take place in this system, but, with appropriate changes of nutrient fluid, cell metabolism continues for several weeks. These cultures are not examined for cytopathic changes, therefore, when used for virus isolation from stools, the problem of nonspecific cytotoxicity, which may interfere with the observation of specific virus-induced cellular changes in tube cultures, does not occur. Passage of the first suspended-cell culture fluid, however, is required before the presence of virus can be established

Certain procedures developed, used, and described in Enders' laboratory[9] are reported below Although they have been largely supplanted, they are described here because of their simplicity and because most of the developments in the field have stemmed from them The preparation of media is outlined in the Appendix

A Preparation and Storage of Tissue

1. *Human embryonic tissues* This material is obtained under sterile precautions at the time of abdominal hysterotomy for therapeutic indications Embryos of between 12 and 18 weeks' gestation are usually obtained Whenever possible the embryo is removed from the amniotic sac under sterile precautions, transferred to a

2. *Maintenance of cultures* Cultures are maintained for periods varying from 2 weeks to 1 month by changing the medium at intervals of 3, 4, or 5 days This is accomplished by removing the medium as completely as possible with a capillary pipette after the tissue has settled. A new pipette is used for each culture On the average, the volume of residual medium is somewhat less than 0.2 ml. Three ml of fresh nutrient are then added to each flask, using a clean serologic pipette for each set of comparable cultures, for example, cultures inoculated with the same material or the same dilution of virus

Between changes of the fluid phase, the metabolic activity of the tissue is reflected by the rapidity with which the pH of the medium declines Immediately after the cultures are prepared the pH is usually about 7 4 In the presence of actively metabolizing tissue, the pH falls to 6 8 or lower during the ensuing 3 or 4 days The pH of each culture is recorded just before the stopper is removed prior to each change of medium

C Isolation of Virus from Clinical Specimens

Suspended-cell cultures may be used on the day of preparation To each of 2 to 4 cultures is added 0 1 ml of stool suspension (or throat swab or spinal cord), treated as indicated on pages 63 to 65 After 5 days of incubation at 36° C , the fluids are harvested and tested in vitro for the presence of virus A series of experiments with stools of patients and with established strains of poliomyelitis virus showed that virus is adsorbed in suspended-cell cultures (of monkey kidney) in about an hour and then multiplies in them to reach peak titers usually in 5 days[34] If the amounts of virus in the original inoculum are minimal, then virus may not be detected in the 5th day harvest Because of this possibility, the fluid should be replaced after the 1st harvest, and a 2d harvest made 5, 10, and 15 days later

D Detection of Virus

1. *By the pH-differential test.* As indicated above, infected tissues usually fail to maintain a metabolic rate, as measured by acid production, comparable to uninfected control tissues To follow changes in pH colorimetrically a series of KH_2PO_4–NaOH buffers are prepared, covering the range from 6 8 to 7 6, with an interval of 0.2 pH unit. The pH of each buffer is checked with a pH meter and phenol red is added in a concentration of 20 μg per ml These standards are sealed in 3 ml amounts in 25 ml Erlenmeyer flasks by drawing out the necks in a gas-

B Preparation and Maintenance of Cultures

1 *Preparation of cultures.** As routine, suspended-cell cultures are prepared in 25 ml. Erlenmeyer flasks Three ml of nutrient are added to each of a series of flasks, which are then loosely closed with No 0 rubber stoppers With a large bore capillary pipette (about 3 mm. internal diameter), 3 or 4 drops of the minced tissue suspension are placed in each flask. With practice, the number of fragments added can be kept fairly uniform by inspection. The flasks are agitated to distribute the tissue in the medium and are tightly stoppered † Unless the flasks are tightly stoppered, the pH of the medium of individual cultures inoculated with the same material may vary widely. Cultures are incubated in the stationary position at 35° C After 1 to 4 days, the first change of medium is made, and the virus inoculum is added. In each test a minimum of 3, and usually 4, cultures are inoculated Three or 4 cultures to which virus is not added are always included as controls.

Ordinary 16 by 150 mm test tubes may also be used for suspended-cell cultures, with 1 to 2 ml of medium, the tubes are kept on their sides at an angle of about 5° so that the tissue fragments are bathed by a thin layer of fluid Tubes are held stationary (or may be rolled) during incubation at 36° C The suspended-cell culture technic has been adapted to 5 l bottles for the large scale production of virus [10]

The nutrient medium may consist of: (1) medium No. 199[8]; or (2) 0 5 per cent lactalbumin hydrolysate in Earle's balanced salt solution[11], or (3) 1 part serum ultrafiltrate and 2 parts balanced salt solution[9] Penicillin (100 units per ml) and streptomycin (100 µg. per ml) are incorporated into the medium to reduce bacterial contamination, and phenol red (20 µg per ml) is included to serve as an indicator of cell metabolism as reflected by pH changes

with embryonic skin-muscle tissue, the cytopathic effect of virus in suspended-cell cultures may become apparent as early as 8 days after the flask is inoculated with virus Plasma hanging-drop cultures are prepared from tissue fragments removed from flask cultures at varying intervals after the addition of virus Control hanging-drop cultures are made at the same time from uninoculated flasks In 2 to 3 days fragments from control uninoculated flasks develop well-defined zones of cells which continue to increase and present a normal appearance for 7 days Fragments from virus-infected cultures either fail to show cell migration or exhibit a meager outgrowth with pronounced degenerative changes occurring within 5 days Tables 2 and 3, taken from Robbins *et al*,[12] show virus titrations and antigenic typing carried out by this method

TABLE 2

TITRATION OF VIRUS IN SUSPENDED-CELL CULTURES BY INHIBITION-OF-CELL MIGRATION TEST*

Cell migration in plasma hanging-drop cultures † Fragments taken from flask cultures 22 days after inoculation, with concentration of virus indicated

Virus strain	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	No virus
Lansing‡	0/6	0/6	1/6	2/6	6/6	10/10
Brunhilde§	0/6	0/6	0/6	5/6	6/6	10/10

* From Robbins, Enders and Weller[12]

† Ratio of numbers of explanted fragments showing normal cell migration after 5

TABLE 3

SPECIFIC INHIBITION OF THE CYTOPATHIC EFFECT OF POLIOMYELITIS VIRUS AS INDICATED BY THE CELL MIGRATION TEST*

Cell migration in plasma hanging-drop cultures Fragments taken from flask cultures 26 days after inoculation of virus (0 1 ml of tissue culture fluids) and serum indicated

Virus Inoculum	Lansing Antiserum	Brunhilde Antiserum	Normal Monkey Serum
Lansing	12/12	0/12	0/12
Brunhilde	0/12	12/12	0/12

* From Robbins, Enders, and Weller[12]

oxygen burner. The pH of cultures is estimated by direct comparison with these standards.

A distinct pH differential between the control and infected cultures may be noted from 12 to 20 days after inoculation The test is not absolutely reliable, however, and subcultures to other flasks should be made if the pH-differential test in the original cultures is negative. Sometimes the test becomes positive only in the subculture. Virus titrations may be carried out by using this test to determine the end point of virus infectivity. In the example shown in Table 1, the suspension had a titer of 10^{-4} per 0 1 ml of the volume of inoculum

TABLE 1

TITRATION OF VIRUS BY THE pH-DIFFERENTIAL TEST IN SUSPENDED-CELL CULTURES OF HUMAN EMBRYONIC BRAIN*

Concentration of Virus†	pH of Culture Fluid‡ after Indicated Number of Days of Incubation						
	3	7	11	15	20	24	28
10^{-1}	6 8	6 8	6 9	7 1	7 2	7 3	7 3
10^{-2}	6 8	6 8	6 9	7 1	7 2	7 3	7 3
10^{-3}	6 8	6 8	6 8	7 0	7 1	7 3	7 3
10^{-4}	6 8	6 8	6 8	6 9	6 9	7 2	7 3
10^{-5}	6 8	6 8	6 8	6 8	6 8	6 8	6 9
No virus	6 8	6 8	6 8	6 8	6 8	6 8	6 9

* From Robbins, Enders, and Weller 12

† Inoculum mouse brain infected with the Lansing strain

‡ Average of individual pH readings of the fluid medium in 3 flasks Supernatant fluids were removed and fresh medium added on the days indicated Readings were made just before this was done The pH of the fresh media varied from 7 5 to 7 7

2 *By the cytopathogenic effect.* Fragments of tissue in which poliomyelitis virus has grown show a necrotic appearance when examined histologically.[12] Few or no well-preserved cells may be seen. The changes consist of loss of typical staining properties, nuclear pyknosis, and fragmentation of cells. Fragments of the same specimen of tissue which have been held in control cultures not exposed to the virus should also be examined for comparative purposes Histologic differences between control tissue and tissue which has supported virus growth become apparent between the 16th and the 30th day of cultivation.

3. *By the inhibition of cell migration.* In this test, which works best

into the nutrient medium has been found useful Synthetic medium No 199 contains a well-buffered mixture of amino acids, glucose, purines, pyrimidines, vitamins, coenzymes, and minerals (see Appendix). If a strong buffering medium is present (Earle's salt solution), the cellular outgrowth may be sufficiently luxuriant for use before any replacement of medium is necessary

Certain sera, particularly bovine, may contain virus inhibitory substances If they are added to stimulate growth, it is essential before the culture is used to replace the serum-containing medium with a maintenance medium free of serum This tedious operation can be overcome by using sera from selected animals proved to be free of inhibitory substances. The sera of 6-month-old calves are highly satisfactory in this regard.

Media containing amniotic fluids, embryonic extracts, and serum may support the continued growth of cells for periods of several weeks, however, cultures containing medium No 199 or lactalbumin hydrolysates without added serum are satisfactory for short term experiments, especially for neutralization tests Moreover, cultures with these media support virus multiplication as well as do those containing embryonic extract or serum For certain purposes the benefits of protein-free fluid media are great, as in the production of virus for vaccines[20] and in complement-fixing antigens[22,23]

For illustration, the following procedures with human tissues have been taken from reports of the Children's Medical Center of Boston[13,14] Similar methods have been described for monkey tissues[15-17] These papers should be consulted for more detailed descriptions of the behavior of tissues in plasma-clot cultures

A. Preparation of Tissues

A variety of tissues of human and monkey origin may be employed Those which have been more extensively studied include human embryonic skin-muscle and lung, human uterine, testicular, or tonsillar tissue, and kidney and foreskin from young children Kidney and testis from rhesus or cynomolgus monkeys may be used Some of these tissues have already been discussed in the section dealing with suspended-cell cultures, and only a few additional comments will be made here Very small embryos may be minced and used in their entirety Uterine tissue is obtained at operations performed for a variety of conditions that include carcinoma of the cervix, adenomyosis, and adeno-carcinoma Material from younger women, ranging in age from 32 to 43 years, who have not reached the menopause is selected Immediately after removal, a section of the wall is taken without entering the lumen, at a point removed from any lesions The tissue, therefore, consists predomi-

E. Antigenic Identification of Virus

Neutralization tests with specific antisera to the 3 types of poliomyelitis virus are usually carried out in roller-tube or stationary-tube cultures, where the results become apparent more quickly than in suspended-cell cultures. They can, however, be performed in flask cultures as follows:

0.1 ml of undiluted antiserum (or 1.5 or higher dilution if the serum is potent) is added to a suspended-cell culture. One half hour later 0.1 ml of pooled fluid from a set of flask cultures is added. Some contain normal monkey serum and virus, and others contain neither virus nor serum. All cultures are incubated at 36° C. Nutrient fluid is changed every 4 or 5 days as needed, and the pH of the medium is recorded. The pH-differential test (see above) becomes positive in the control virus cultures and in those cultures containing heterotypic antisera; that is, their metabolism is inhibited so that cultures in which virus grows do not become as acid as cultures without virus, or cultures containing virus and homotypic antibody. The inhibition of cell migration for virus activity may also be neutralized by type-specific antiserum, as shown in Table 3. The virus is identified as to type by the serum which neutralizes it.

III. ROLLER-TUBE TISSUE CULTURES

Such cultures may be prepared by embedding tissue fragments in an avian plasma clot spread thinly over the inner wall of a 16 to 18 mm diameter test tube to which 1 to 2 ml of nutrient medium are then added. The test tubes are placed in a drumlike, perforated holder, which is itself mounted on a spindle at its center. The entire unit is rotated (about 12 revolutions per hour), thus cyclicly bathing the tissue embedded in the plasma-lined tubes. As with flask cultures, the fluid medium is usually replaced every 4 or 5 days, or at longer intervals if the medium is well buffered. After several days at 36° C., proliferation of fibroblasts or epithelial cells is usually quite marked. If poliomyelitis virus is now added to the cultures, subsequent cytopathic changes in the outgrowth serve as an *in vitro* indicator of virus multiplication, which can be observed and followed readily under the microscope.

The nutrient media used in plasma-clot tube cultures of poliomyelitis virus may contain whole serum, serum ultrafiltrate, embryonic extracts, bovine amniotic fluid, and buffered salt solution.[12-17] For some purposes, the incorporation of 0.5 per cent of enzymatic hydrolysate of lactalbumin or other proteins[11,18] or synthetic medium No. 199[19-21]

clear areas occasioned by plasmolysis or retraction appear in the plasma coagulum in the vicinity of the tissue fragments The migrating or growing cells tend to be compressed along the margins of these areas, and the growth of fibroblasts into them is restricted As interruption of the plasma layer extends, many of the tissue fragments may become detached, leaving behind only small aggregations of fibroblasts Damage to the plasma coagulum does not interfere significantly with the growth of epithelial cells, since in contrast to fibroblasts they tend to adhere to the glass With the exception of kidney and prepuce, the outgrowth from the tissues mentioned has been predominantly fibroblastic When lysis begins to appear, the plasma coagulum may be repaired or "patched" in the following manner The nutrient medium is removed A drop of plasma is added and distributed over the surface of the culture by rotation of the tube A drop of chick embryo extract is then spread in the same way Actively growing cultures of human fibroblasts may require "patching" every 4 to 5 days

The need for this time-consuming manipulation may be eliminated through the use of crystalline soybean trypsin inhibitor,* commercially available A concentration of 0 18 mg per ml in the medium practically eliminates lysis of cultures of human embryonic skin-muscle tissue A concentration of 0 05 mg per ml, however, so delays disturbance of the coagulum that "patching" is unnecessary, unless the cultures are maintained for an unusually long time A 1 per cent stock solution of the inhibitor in balanced salt solution may be prepared, sterilized by filtration, and stored at 4° C until used

C Isolation and Typing of Virus

1 *Isolation of virus* For virus isolations in plasma-clot cultures of human tissue, embryonic skin-muscle, mature uterus, or kidney tissues are used [21 25] Cultures are nourished with 2 ml of a medium containing 90 per cent bovine amniotic fluid, 5 per cent bovine embryonic extract, and 5 per cent horse serum [14] The following procedure is recommended by Kibrick and others [25] a 10 per cent fecal suspension is made in the bovine amniotic fluid medium, which for this purpose contains 100 units of penicillin and 50 μg of streptomycin per ml The suspension is centrifuged in the cold at 4,000 r p m for 1 hour 0 1 ml of the supernatant fluid is inoculated into each of two cultures (The remainder of

* Worthington Biochemical Laboratory, Freehold, N J

nantly of myometrium The specimen is wrapped in sterile gauze moistened with saline for transfer to the laboratory After mincing, as described in the section on suspended-cell cultures, tissues are washed several times with balanced salt solution.

Treatment of kidney tissue with trypsin as recommended by Simms for adult tissues may reduce the period before cell outgrowth occurs. This procedure is carried out in the following manner minced tissue is incubated for 2½ hours at 35° C. in 2 or 3 volumes of 0 5 per cent solution of trypsin (1 250) made in salt solution-ultrafiltrate mixture and sterilized by filtration The pH of this solution should be previously adjusted to 7 4 to 7 5 by addition of 1 4 per cent sodium bicarbonate solution After incubation, the tissue fragments are washed 3 times with nutrient and then explanted If not immediately explanted, about 2 ml of tissue fragments may be suspended in 20 ml medium and stored in 250 ml. stoppered flasks at 4° C for varying periods of time They have proved viable after storage for 2 weeks or longer

B PREPARATION AND MAINTENANCE OF ROLLER-TUBE CULTURES

1. *Preparation.* One or 2 drops of chicken plasma are spread in a thin layer on the lower two-thirds of the tube with a capillary pipette or sterile swab The tube is slanted, and any excess plasma removed after it has collected in the bottom. With a capillary pipette, about 2 mm in diameter at the tip, 1 drop of the tissue suspension is deposited on the wall of the tube and the fragments distributed as uniformly as possible in the thin plasma layer During the preparation of the cultures, the fragments are suspended in a very small volume of nutrient, since excess fluid tends to prevent their adherence to the wall of the tube. Immediately after the introduction of the tissue, 1 drop of chick embryo extract is added, and the tube is rotated in order to achieve even distribution of the extract throughout the plasma layer Chick embryo extract is employed to provide homologous thromboplastin, since firm clotting of the plasma does not occur in the presence of the heterologous thromboplastins supplied by the human tissue or by the beef embryo Commercial thrombin has been used, however After clotting has taken place, 2 ml. of the nutrient medium are added to the tube, which is then stoppered tightly and placed in the rotating drum for incubation

2 *Maintenance* Every 2 to 3 days, the cultures are removed from the rotating drum, the fluid medium is withdrawn, and the same quantity of fresh medium added The progress of cell growth is followed by examining them under a magnification of 50 to 100 times The culture tubes are supported on the microscope stage in a slightly inclined position in order to prevent the fluid from touching the stopper. After 5 to 7 days

a. Indirect method A 1:10 dilution of fluid from a roller-tube culture showing cytopathic changes provides the virus for typing. To 0 15 ml of each prototype serum in separate tubes is added an equal quantity of virus dilution The tubes are shaken, incubated for one hour at about 4° C., and 0 2 ml. of each mixture is then transferred to separate roller-tube cultures The roller-tube medium is changed prior to inoculation but only as needed thereafter Cytopathic changes are usually evident in two of the three cultures within 2 to 5 days, depending on the tissue employed The type of the unknown virus is indicated by the prototype serum in the intact, remaining culture Rarely, all three tubes may show evidence of viral degeneration In such cases the typing is repeated with a more dilute viral inoculum

b. Direct method For direct typing the method is similar to that employed above except that the 10 per cent fecal supernate is substituted for the first tissue-culture passage fluid as the virus source. The medium is changed on the following day Further addition of antiserum, however, is not necessary While any tissue may be used, human kidney is the tissue of choice because of its greater sensitivity, more rapid response to the presence of small amounts of virus, and greater resistance to fecal toxicity With tissue cultures of both human and monkey kidney, the use of larger fecal inoculum and one hour periods of contact with the culture are also feasible for direct typing at the time of virus isolation.

IV STATIONARY-TUBE CULTURES

Satisfactory results may be obtained with tube cultures which are held stationary, as well as with those which are rolled [11,17,18,21] Cultures are prepared as for roller tubes, using 16 by 150 mm test tubes They are held stationary on their sides slightly inclined in a rack (5°), so that 1 ml of nutrient fluid forms a shallow layer and reaches about one-third of the distance from the bottom of the test tube to its mouth (Fig 4) Several tissue fragments are submerged in the fluid medium The cultures are incubated at 36° C and are treated as outlined above for roller-tube cultures As with roller tubes, stationary-tube cultures are useful for isolating and typing poliomyelitis viruses and for carrying out neutralization tests.

Stationary-tube cultures can be prepared in the absence of a plasma clot, for tissue fragments can be made to stick on glass preheated to 45° C in the absence of plasma and clotting solution [21] The cultures are

the supernatant fluid is frozen and stored at −15° C.) The medium is replaced the next day and again as required when the pH falls If no cytopathic changes characteristic of viral activity are observed after 4 to 5 days of incubation at 35° C., 1 to 3 ml of the fecal suspension are thawed and inoculated into new tissue culture tubes The cultures are rotated at 35° C for an hour The fluid contents are then replaced by 2 ml. of fresh medium, which are again changed on the next day. If the toxic effect* of the fecal suspension is marked at 24 hours, the cultures are allowed to incubate for another day, and 0.1 to 1 0 ml. of the culture fluid is inoculated into fresh cultures Where the toxic effects are not pronounced, the cultures are observed daily thereafter for cytopathic changes induced by the virus Figures 1, 2, 3 show virus-induced changes in fibroblast and epithelial cell cultures, as seen in living preparations under the ordinary, and the phase, microscopes.

Uterus, embryonic skin-muscle, testis, and foreskin cultures should be examined for two weeks after addition of the specimen Virus-induced degeneration proceeds more quickly in kidney cultures, and such cultures need be observed for only one week Cultures of kidney tissue have proved better able to withstand larger volumes of fecal suspension than cultures of other tissues, since kidney epithelial cells appear to be more resistant to fecal toxins Moreover, such cultures are not seriously affected by the trypsin-like activity which many fecal suspensions exhibit and which cause lysis of the plasma coagulum, thus resulting in the removal of growth completely Since kidney epithelial cells are mainly attached to the glass, they remain undisturbed by the disintegration of the coagulum

2 *Typing of virus* The following procedures have been used by Kibrick, Enders, and Robbins[25] Immune sera, prepared in monkeys (*see* Appendix) *are inactivated at 56° C for 30 minutes* and diluted to 1 3 prior to use. Saline or phosphate buffer may be used for preparation of serum and virus dilutions.

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* Toxic effects vary from cellular granularity to rounding and necrosis Cells or tissue fragments may be liberated in the fluid medium

The technic is relatively simple, requiring only test tubes,* preheated at 45° C and stored in the boxes in which they have been washed and sterilized, an empty box for transfer of the tubes, an uncalibrated pipette (tapered to a 2 mm opening) with small rubber bulb, 2 flat racks for holding 24 tubes, and the tissue mince The warm tubes from the box are placed on the rack, and kidney fragments are streaked in a narrow line into the lower third of all 24 tubes For best results the tissue, enough for 24 tubes, is taken into the pipette with the minimum amount of fluid necessary to make the streaking possible After the tubes have had tissue put into them, they are transferred into another box The preparation of a box of 170 tubes in this manner takes about 5 minutes During this time there is a drop in temperature of the tubes from the original 45° C Adhesion to the tubes or growth potential of the tissue does not vary discernibly in the first or last tubes made in each box When the tubes have all been placed in the second (transfer) box, the sterile top from the first box is also transferred to it These tubes remain at room temperature for about 5 minutes while the next box is being prepared After this time, they are transferred to a refrigerator (4° C) where they remain for 30 minutes (It is detrimental to hold them without fluid for more than 60 minutes, even at 4° C) After their temporary storage in the cold, the tubes are filled with 1 ml of growth medium by an automatic Cornwall pipette † When added to the tube, the fluid should not be directed on the tissue fragments The tubes are quickly stoppered after addition of medium and are held in boxes similar in size to those described above The storage box has a board strip 256 mm by 10 mm by 5 mm high, fixed across the front lower edge to provide the desired tilt to the tubes A row of small nails with 4 mm heads driven into the back of the box 16 mm apart and 12 mm from the bottom of the box keeps the first row of tubes in position and facilitates the storage of the upper rows Each box firmly holds 145 tubes Each alternate tube is pulled about ½ inch to reduce excessive tilting of the upper rows, which results from the diameter of the stoppers being larger than that of the tubes The tubes are left *undisturbed* at 36° C for 10 to 14 days, at which time clear, luxuriant sheets of epithelial cells about 10 mm in mean diameter appear Not every tissue fragment gives rise to a sheet of cells, but usually 2 to 4 sheets are formed in each tube Before use, the growth medium is replaced with a maintenance medium consisting of 1 part of No 199 and 1 part of Earle's salt solution

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* *Washing and care of test tubes* Test tubes (16 by 150 mm) are immersed over

incubated at 36° C., and the new cells grow out directly on the glass surface. No antitryptic factor is necessary to prevent the lysis of the clot and the possible loss of the culture Before the culture is used, the tissue fragment, easily removed to leave behind a sheet of newly grown cells, can be transferred to seed another culture tube. This technic has been used chiefly with monkey kidney tissue.

Production of virus in large quantities, as required for production of vaccines or complement-fixing antigens, is possible, using sheets of epithelial cells of normal monkeys, bathed in protein-free media at the time of virus inoculation. In these preparations no proteins incorporated in the plasma clot can contaminate the virus yield Virus can also be grown in suspensions of kidney fragments without added protein, but the epithelial outgrowth appears not only to be more sensitive to smaller amounts of virus, but also to yield fluids of higher virus titer.

A. Preparation of Tube Cultures

Kidneys are taken from either cynomolgus or rhesus monkeys Soon after its removal from the monkey, the kidney is minced in a wide-mouth tube (about 1 inch) containing two volumes of Earle's salt solution. The kidney is reduced to fragments of about 1 mm. in diameter and the suspension of fragments is transferred to a 100 ml. flask. They are washed by repeated addition of salt solution and decantation of the turbid supernate The heavy fragments settle rapidly and the lighter undesirable fractions can be removed, using about 300 ml. of salt solution per kidney Kidney tissue thus washed is kept in a 100 ml. flask in 50 ml of Earle's salt solution at 4° C until needed. It can be used for as long as 3 days with no appreciable loss of viability. One pair of kidneys from a young monkey (5 to 7 pounds) can provide tissue for 2,000 primary culture tubes

The growth medium consists of 90 per cent medium No 199 and 10 per cent bovine serum The use of serum from 6-month-old calves is recommended, for virus inhibitory substances often present in the serum of adult animals are not usually found in such calf serum. Penicillin and streptomycin are added so that the final concentration is 100 units and 100 μg. per ml. respectively. Antimycotic agents such as nystatin at concentrations of 100 units per ml. may also be incorporated into the medium; 1 ml. of nutrient is added to each culture

D. Identification of Viral Types by Neutralization Tests

Fluids from primary isolation, or from second passage cultures, showing a viral type of degeneration are tested against each of the three specific poliomyelitis antisera The sera are prepared by hyperimmunization of monkeys (see Appendix) and should have titers of more than 1 1,000 against 100 TC_{50} doses of virus The test is carried out as follows[15,28]. 0 2 ml of tissue culture fluid, 10-fold diluted, is mixed with 0 2 ml of each antiserum, previously diluted 5-fold with balanced salt solution. After incubation for 1 hour at room temperature, 0 1 ml of each serum-virus mixture is inoculated into 2 kidney culture tubes The cultures are incubated in a stationary position until examined, 3 and 6 days later The virus is identified by the serum which neutralizes its cytopathogenic property If a virus is not neutralized by any of the three antisera, it is tested again by carrying out titrations of the virus in the presence of each of the antisera Occasionally a poliomyelitis virus of high titer may not be neutralized if undiluted or if 10-fold diluted tissue culture fluid is mixed with antiserum

As with roller cultures, isolation and typing may be accomplished at the same time, and this is the most rapid method for typing viruses in clinical specimens The material under test, usually stool suspension, is *mixed with an equal volume of 1 10 dilution of type-specific poliomyelitis antisera* The suspensions are then tested for virus as outlined in the above Section C Virus degeneration will occur in cultures inoculated with the stool plus heterotypic sera, but not in cultures receiving the stool plus homotypic serum

V TRYPSINIZED KIDNEY-CELL CULTURES

Dulbecco and Vogt have reintroduced the technic of growing cells obtained directly from trypsinized tissue on glass surfaces[26,29] The cell suspensions which are prepared from kidney fragments produce luxuriant monolayer cultures on any clean glass surface, petri dish, test tube, or bottle held in the stationary position Tube cultures prepared in this way are used in the same way as cultures prepared as outlined in the above sections

Poliomyelitis virus will give rise to plaques on monolayer cultures of epithelial tissue obtained by the technic introduced by Dulbecco. Counting plaques permits highly accurate virus and antibody titrations, *and pure lines of virus can be isolated from single plaques* The pro-

B. Preparation of Bottle Cultures

The same technic employed in making tubes can be used in growing plasma-free cultures in flat-sided *bottles*, from 1 to 5 l in volume The amount of tissue needed varies with the size of the bottle For example, with a bottle having a flat side of 36 sq cm, 4 or 5 drops of minced kidney suspension are placed on the inside wall and spread about with a pipette The mince of a third of a kidney may be placed on the flat side of a 5 l culture bottle The bottle is stoppered and turned to allow excess fluid to drain from the tissue After 5 minutes at room temperature and ½ hour at 4° C, the growth medium is added to cover the tissue with about 7 mm of fluid The bottles are incubated in the stationary position at 36° C. The growth rate of monkey kidney tissue in bottles parallels that of test tube cultures

Prior to inoculation of bottle cultures, tissue fragments are manually shaken loose from the wall of the bottle The fragments and the media in which they are suspended are transferred to another sterile bottle and incubated further at 36° C. The original bottle is rinsed thoroughly with salt solution to wash out the serum, and maintenance medium is added to cover the tissue with about 7 mm. of fluid Bottles with confluent growth inoculated with 10,000 to 100,000 TC_{50} doses of virus have yielded harvests 24 to 48 hours later with titers of $10^{-6.5}$ to $10^{-7.5}$, and at times even 10^{-8} per ml.

C. Isolation of Virus in Kidney Epithelial Outgrowth

Material suspected of containing virus is treated to remove bacteria as outlined in the chapter on Poliomyelitis. To each of 4 culture tubes, 0.1 ml of the treated suspension may be added and the tube maintained stationary at 36° C. Tubes are examined daily for 10 days to determine whether material such as stools are toxic for cultures (usually apparent within 24 hours) or whether they contain a cytopathogenic agent (usually apparent between the 3d and 5th days) If stools are cytotoxic, fluids for passage are harvested on the 5th day, otherwise they are harvested when degeneration is clearly evident.

Because virus is rapidly adsorbed to tissue culture cells, larger amounts (1 ml) of material to be tested may be added to the cultures, removed after an adsorption period of 1 hour, and replaced with maintenance medium The period of 1 hour is sufficiently long for adsorption of a large part of the virus but is short enough so that cytotoxic effects are rarely noticed. Epithelial cellular outgrowth from kidneys is more resistant to the toxic factors in stools than is the fibroblastic outgrowth from testis.

Passage and neutralization tests are carried out to identify the virus.

Step 3 The cut tissue is transferred to the trypsinization flask (Fig 5)* and covered with trypsin prewarmed to 37° C

Steps 1 to 3, the preparation of 1 kidney, should take about 7 to 10 minutes The process is repeated for each of the remaining kidneys After each kidney is prepared it is added to the others, covered with trypsin, and incubated Thus, 6 to 8 kidneys may be conveniently prepared in the hour required before stirring begins

Step 4 One hour after trypsin is added to the 1st kidney the incubation fluid is decanted into a sterile flask, which is kept at 4° C Fresh prewarmed trypsin is added to the flask to a level equal to a total volume of about 150 ml

Step 5 Tissue and trypsin are stirred with a magnetic stirrer for 7 minutes The speed of stirring should be sufficient to allow rapid mixing with cavitation but no foaming

Step 6 After 7 minutes of stirring, the magnetic stirrer is turned off, the tissue allowed to settle, and the fluid decanted into the receptacle containing the incubation fluid

The remaining tissue is washed clean of free but adhering cells by adding 50 to 100 ml of trypsin solution, and then swirled by hand and decanted If the tissue has been solubilized significantly during a run or wash, the decanted fluids when held to the light are seen as a fine suspension of tissue fragments and cells After the 3d or 4th run, it may be necessary to wash the tissue 2 to 4 times before the washings are clear

This step, decanting, washing, and refilling, should be done without haste and may take as much as 5 to 10 minutes

Step 5 (mixing with trypsin) and Step 6 (decanting and washing) are repeated 6 to 8 times or until there is a noticeable decrease in the rate of solubilization of tissue as indicated by a clearing of the fluids

Step 7 After 6 to 8 runs, when the fluids have noticeably cleared, the tissue is removed to a small beaker Usually at this point considerable connective tissue has become visible during trypsinization and may interfere with mixing The connective tissue is cut and if necessary the remaining tissue should be cut into pieces of 0 5 cm in size The tissue is covered with trypsin and incubated at 37° C for 20 minutes

Steps 5 and 6 are repeated after the 2d incubation until tissue is exhausted The number of runs required to exhaust tissue varies with the mass of tissue A total of 8 runs is usually sufficient for 4 kidneys and 10 to 13 for 8 kidneys

Step 8 The combined fluids from all of the runs are centrifuged at 200 r p m [illegible]

Step 9 The supernatant fluid is removed from the packed cells by a vacuum aspirator The fluid can be removed to within 1 to 2 ml without removing cells

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* The flask, as well as the automatic type shown in Figure 6 may be obtained from Macalaster Bicknell Co, Henry St New Haven Conn or from Microbiological Associates, Bethesda, Md

cedure below is Rappaport's modification[35,36] of the Dulbecco-Vogt-Youngner[27] method.

A Solutions, Media, and Cell Counts

1 *Phosphate-buffered saline (PBS)*

a NaCl 8 0 gm , KCl 0 2 gm , Na_2HPO_4 1 15 gm , KH_2PO_4 0 2 gm ; water 800 ml

b $CaCl_2$ 0 1 gm , water 100 ml

c $MgCl_2 \cdot 6H_2O$ 0 1 gm , water 100 ml

Autoclave solutions a, b, and c separately and mix when cool

An alternative method is to dissolve all the salts in 1,000 ml and to sterilize by filtration

2 *Trypsin*, 1 250 or 1 300 Prepare a 0 20 per cent solution in PBS Sterilize by filtration

3 *Growth medium* For washing, suspending, and growing the dispersed cells, the following medium is used lactalbumin enzymatic hydrolysate, 0 5%, calf serum, 2 0%, Hanks' solution, 97 5%

4 *Cell counts* One part of cell suspension is treated with two parts of a stain of 0 1% crystal violet in 0 1M citric acid The stained suspension is mixed thoroughly and counted, using a hemocytometer All the cells falling in the four large squares of the hemocytometer, as used in a white cell count, are counted and averaged Cell count per ml equals average count per square $\times$ 10,000 (hemocytometer volume) $\times$ 3 (dilution factor) Only cells showing both nuclei and cytoplasm are counted The following convention is used in counting clumps clumps in which the individual nuclei are surrounded by a large volume of cytoplasm are treated as aggregations of single cells and every visible cell is counted When the clump shows the nuclei close together with little visible cytoplasm, the whole clump is counted as only one cell Three aliquots of the suspension to be counted are taken and stained The counts on each aliquot are made in duplicate, including charging the hemocytometer, and averaged

B. Preparation of Trypsin-Dispersed Cell Suspensions

The preparation and trypsinization of 8 kidneys takes about 4 to 5 hours The yield is between 0 6 and 0 8 $\times 10^8$ cells per gm of "dressed" tissue

Step 1 Kidneys are removed from freshly killed and exsanguinated monkeys (rhesus, cynomolgus, cercopithecus, and baboons may be used) The kidneys are decapsulated and cut in half This is best done by holding the kidney firmly with forceps and making a cut through the kidney horizontal to the flat surface with uterine scissors, exposing the central pelvis The pelvis is removed by cutting around its outer edge, lifting it with forceps to free it from the underlying tissue Any adhering connective tissue is also discarded

Step 2 The tissue is cut into pieces about 1 cm by 1 cm It is important to cut through the tissue because strands of connective tissue may interfere with proper mixing

1. Operation of flask The tissue and magnet are placed in the flask in which the outlet drain has been closed by a pinch clamp 100 to 150 ml of trypsin solution, prewarmed to 37° C, are also added The trypsin reservoir is placed so that trypsin can be admitted into the mixing chamber by siphon action through a drop of at least 1 foot The reservoir should be kept at 37° C The outlet drain should feed into a suitable receptacle, placed at least 1 5 feet lower than the mixing chamber. If it is desirable to tap the trypsinizer for cell suspension throughout the day, it is convenient to use a bell cap on the outlet tube for the receiving flask. The bell cap can be changed easily from one receptacle to another with a minimum chance of contamination If the cells are to be kept several hours before harvesting, the receptacle should be kept in an ice-water bath

The magnetic stirrer is started and the flask centered so that the magnet operates smoothly in the center of the flask The tissue is incubated with gentle stirring for 30 to 45 minutes After incubation, the speed of the stirring is increased until there is rapid swirling of tissue up and around the sides of the mixing chamber There should be slight cavitation but no foaming

At this time, with the air valve closed, the drain valve is opened slowly. Trypsin will flow from the mixing chamber into the receiving jar and will prime the siphoning of trypsin from the reservoir When trypsin starts flowing into the mixing chamber, the inlet valve should be closed gradually until, with the drain valve *completely open*, the rate of flow through the flask is regulated by the pinch clamp on the inlet from the trypsin reservoir

During trypsinization, small pieces of tissue may escape through the drain into the receiving jar If the tissue has been cut as directed and if the speed of the magnet is properly adjusted, the tissue actually lost in this way is negligible If the flow of fluid is regulated, however, by a pinch clamp on the drain valve instead of on the trypsin inlet as indicated, the constriction will tend to trap any tissue escaping and may block the outflow of fluid

The yield of cells per gm of kidney has been found to be somewhat greater than by the manual method, that is, 10^8 cells per gm as compared with 6 to 8 $\times 10^7$ per gm obtained by the manual method

Two to 3 l of trypsin are sufficient for 4 kidneys, the manual method requires 3 to 4 l

D. Preparation of Cultures

Standardized dispersed cell suspensions are distributed into various types of culture vessels by means of a Cornwall automatic pipetting unit Uniformity of the suspension is maintained during distribution by the use of a magnetic stirring device or by manual swirling of the flask containing the reservoir of cells.

1 *Culture tubes* are prepared by adding 0 5 ml. of suspension (approximately 150,000 cells) to 16 by 150 mm. test tubes which are rubber-stoppered. These are incubated in a stationary position at a slight tilt from the horizontal position at 36° C. for 6 or 7 days. At the end of this time a zone of confluent kidney epithelium covers the area of the

This residual fluid has not been found to affect the quality or viability of the suspension in any way. There should be approximately 3 ml of packed cells for each kidney trypsinized

Step 10 The packed cells are resuspended in about 100 ml of the growth medium prewarmed to 37° C If cold solution is added, the cells may clump badly The suspension is filtered through 2 layers of sterile cheesecloth The gauze filter is washed free of adhering cells with about 100 ml of nutrient solution

Step 11 The number of whole cells, that is, those showing both nuclei and attached cytoplasm, are counted in a hemocytometer

Step 12 The suspension is diluted in the nutrient medium to give 300,000 cells per ml, and 0 5 ml of this suspension is seeded into 16 by 150 mm tubes, and correspondingly larger volumes for larger vessels After 4 to 5 days of incubation in a stationary, inclined position, a confluent sheet of cells is present.

Step 13 If the suspension is to be kept several days before use, it should be stored at 4° C at a concentration of not more than 600,000 cells per ml The stored suspension should be centrifuged after the 1st 10 to 24 hours and resuspended in fresh growth medium for the rest of the storage period The suspension is again centrifuged and again resuspended in fresh medium just before use (This procedure is necessary for maximum survival and growth because it has been found that freshly trypsinized kidney cell suspensions liberate a heat labile toxin which kills slowly at 4° C and may destroy up to 90 per cent of the cells in a few hours under growing conditions at 37° C) Cell suspensions stabilized in this manner can withstand intercontinental shipping[36]

C. Continuous Automatic Trypsinizing Flask[35]

A flask permitting the continuous and automatic addition of trypsin and withdrawal of cells is shown in Figure 6 It consists of a glass mixing chamber closed by a ground glass joint containing two openings Through one, trypsin can be admitted from the reservoir The other is a valve that can admit air when desirable In the bottom center of the flask, holes have been drilled so that fluid can drain from the mixing chamber into a receiving jar When tissue fragments are stirred in the mixing chamber, the motion of the magnet sweeps the fragments from the drain and permits only the cell suspension to pass through into the receiving jar

The mixing chamber and magnetic stirrer should be arranged so that trypsin is admitted by siphon action through a gum rubber lead The flow of trypsin is thus virtually independent of the amount of trypsin in the reservoir and can be controlled simply by a pinch clamp on the rubber lead into the mixing chamber The drain valve must never be opened unless the magnet is in motion If it is opened and the magnet is not turning, the tissue will settle and clog the pores If this happens, the cells are not recovered as soon as they are released so that they may be lost by excessive digestion with trypsin

[illegible]

for this purpose Operated at proper speeds and on a steady line the trypsinizer can be left trypsinizing overnight unattended

in the absence of serum, may be used for physiologic studies under defined conditions

The cells are harvested from the trypsinization fluids by centrifugation at 200 rpm for 30 minutes and resuspended in 500 times their volume of phosphate buffered saline (PBS) The washed cells are resuspended in 100 to 200 ml of the synthetic medium, filtered through 2 layers of cheesecloth, and diluted to 350,000 cells per ml for seeding of stationary cultures

Cells seeded in SM-1 attach more slowly to the glass By the 3d day, however, the outgrowth in SM-1 compares favorably in both amount and quality with the suspensions seeded with the serum-containing lactalbumin hydrolysate (M) medium of Melnick[42] (*see* Appendix) The two sets of cultures are comparable up to the 10th to 12th day after leaving the monkey The M medium must be changed between the 5th and 7th day because of the decline in pH It is not necessary to change the SM-1 medium before the 8th to 10th day

The choice of medium for replenishment is determined by the studies to be undertaken Replenishment of cells grown in SM-1 with the M medium results in further proliferation of the cells after a lag period of 2 days, and survival of a month or more For studies uncomplicated by the addition of serum, replenishment with an amino-acid mixture such as that given in the Appendix, in the same basic salt solution, gives a useful additional lifetime of 1 to 3 weeks

Cysteine is a necessary and specific requirement for successful outgrowth under the conditions studied It has not been possible to replace it with inorganic reducing agents or with other organic sulfur compounds The concentration of salts, particularly calcium, has been found to be a critical factor for outgrowth in the absence of serum Both ferric and cupric ions have pronounced beneficial effects It was also found that if the pH of the medium is allowed to fall below 7.2 to 7.3 during the 1st 4 days, outgrowth is inhibited, cells fail to attach to the glass, and the areas of cells already proliferating along the glass become thickened and granulated Attachment to the glass is also retarded when the medium is more highly fortified with amino acids, phosphates, and traces of certain carboxylic acids or after prolonged metabolism The morphologic changes and lack of attachment are completely reversible Cells have been held in suspensions at 37° C for several weeks and then, after an increase of the pH to 7 6 to 7 8 or after the addition of calcium or zinc, found to attach and grow into luxuriant clear monolayers Since cells held in suspension in this way can synthesize poliovirus, the method has proved useful in following various phases of virus synthesis[61]

1 *Virus growth*[61] The yield of virus propagated in cells grown in SM-1 medium and inoculated and transferred to the amino-acid supplement is the same as that of the control cultures grown and infected in the presence of the serum-containing M medium The propagation of virus in the amino-acid supplement is somewhat faster than that in the M medium and is more striking when the cells have been previously grown in the serum-containing medium The cells grown in SM-1, *and after infection transferred to Hanks' salt solution alone, support the* synthesis of only the 1st cycle of virus, whereas the synthesis of poliovirus continues,

tube in contact with the cell suspension, and the pH of the medium has fallen to about 6.8 By this method, 4,000 culture tubes can be prepared from the tissue of a single monkey It has been observed that almost all of the tubes prepared in this manner are suitable for virus assay or other studies. Before use, the medium is changed to one in which the Hanks' salt solution is replaced by Earle's solution, because of its stronger buffering capacity

2. *Large cultures* may be prepared in flat-sided prescription or Roux bottles. Each bottle receives enough cell suspension to cover its side to a depth of a few mm. After an incubation period of 5 to 7 days, a confluent monolayer of kidney epithelium covers the entire flat glass surface

3 *Petri dish (50 mm) cultures* are prepared by adding 4 0 ml of cell suspension to each dish Incubation is carried out in a well-humidified incubator at 36° C in a continuous flow of air containing 3 per cent CO_2, the mixture being saturated with water The rate of flow should be about 3 l. per minute Fluid is removed after 3 or 4 days of incubation, and 4 0 ml of fresh medium are added On the 6th or 7th day of incubation, the surface of each petri dish is covered with a confluent monolayer of epithelial cells (about 2 million cells).

Cells may be grown in petri dishes in an ordinary incubator in the absence of CO_2 if an organic buffer, Tris (hydroxymethyl aminomethane), at a concentration of 0.01 M (1.2 gm. per l.), is used in place of the bicarbonate buffer.[28] When this buffer replaces the bicarbonate in the lactalbumin growth medium (*see* Appendix), monkey kidney cells produce confluent epithelial cells within a week

E. Cell Growth in Synthetic Medium

The SM-1 medium of Rappaport[61] supports the growth of trypsinized monkey kidney cells in the absence of any added protein Medium SM-1, which is described in the Appendix, consists of *(a)* a salt solution containing a higher concentration of calcium than is widely used, *(b)* trace elements, and *(c)* the organic constituents cysteine, isoleucine, and d-ribose The buffering capacity of the medium is increased by the addition of 8×10^{-3} M Tris (hydroxymethyl aminomethane). It may be supplemented with penicillin and streptomycin if desirable. The medium is stable for at least 1 week at 4° C, and is not used once a precipitate forms on standing The medium cannot be frozen. After outgrowth, the cells support the synthesis of poliovirus and, adjusted to in vitro life

stitution of the simple lactalbumin hydrolysate medium [42] for the more complex media like No 199, and (4) the "sealing" of the panel cups with a paraffin oil of high viscosity.

1 *Materials*

a The diluent for serum and virus is 0 5 per cent lactalbumin hydrolysate in Hanks' salt solution (*see* Appendix)

b The nutrient medium used for cell suspensions is prepared by adding 2 per cent calf serum to the diluent above

c Versene solution for obtaining suspensions of passage cells grown on glass (*see* Appendix)

d Dye-fixative for counting cells 0 1 per cent crystal violet in 0 1 M citric acid.

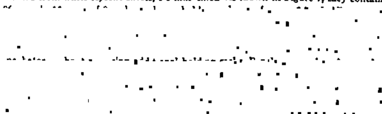
e Paraffin oil (Drakeol No 35, Pennsylvania Refining Co., Butler, Pa) is used to "seal" the panel cups It is routinely autoclaved

f The Cornwall continuous pipetting unit (No 1251, Becton, Dickinson and Co , Rutherford, N J) serves to distribute diluent, virus, cell suspensions, and oil in all replicate operations A 4-inch, No 13 hypodermic needle, whose point has been cut off, makes a suitable cannula, especially when bent to a slight curvature For oil, a specially large cannula is recommended (obtained from Rathgeber Laboratories, 226 County St , New Haven, Conn)

g A cell dispenser may be prepared by making 4 symmetrically placed indentations about the bottom of a liter Erlenmeyer flask and adding 2 ports through which the cell suspension is to pass to 2 Cornwall units, for simultaneous addition of cells to 2 panels The indentations, which increase mixing efficiency, are like those of the trypsinization assembly shown in Figure 5 The ports are introduced at the side of the flask as near to the bottom as is practicable so that the cell suspension may be drained without waste

h Nine-inch lengths of household aluminum foil from rolls 12 inches wide are autoclaved These sterile foil "covers" are used to protect filled cups against airborne contaminants Furthermore, when panels are covered, it is possible to stack them without wetting the bottom of one with the content of the panel below

i Panels (*Linbro Chemical Co , 681 Dixwell Ave , New Haven, Conn*) are molded from white styrene sheets, 0 5 mm thick As shown in Figure 7, they contain



at the conclusion of the readings and are then discarded.

2 *Procedure*

a Cell suspensions: preparation and handling Eighty ml. of primary trypsinized cells (see above) containing 200,000 cells per ml of nutrient medium are placed

although slowly, in the cultures which had previously been grown in the complete M medium

The susceptibility of the two types of cells to poliovirus has been studied by the plaque technic as adapted to flat prescription bottles[53] Because of the sensitivity of the cells to neutral red, particularly in the alkaline range which is required for the synthesis of poliovirus and the maintenance of cells in synthetic medium, it has been necessary to adjust the pH, the buffering capacity, and the time and quantity of neutral red added, in order to achieve the most sensitive and reliable counts Cells grown in SM-1 medium compare favorably with those grown in M medium as regards susceptibility to poliovirus With cells that are grown out in the M medium, the appearance of plaques is significantly faster when the overlay contains the amino-acid supplement rather than serum protein

Serum has heretofore been considered an absolute requirement for the outgrowth of cells The fact that cells may be grown out and maintained for 2 to 3 weeks in the absence of serum suggests that its role is not predominantly nutritive during this period It is possible that serum is largely protective, serving as an effective buffer to maintain the particular physicochemical environment necessary for outgrowth

F The pH Color Test for Neutralizing Antibodies

Suspensions of monkey kidney cells may be used directly as host cells in neutralization tests[37–39] The same basic principle which underlies any virus neutralization test, namely, that antibody specifically neutralizes the infectivity of the virus, also applies to the pH antibody test. The color test employs known quantities of cell suspensions which are inoculated into test tubes or plastic panel cups 1 hour after inoculation of the serum-virus mixture. This eliminates the need for cultures in which cells have already grown out on glass. The color test utilizes the fact that with continued cellular growth in the presence of an immune serum-virus mixture, acidic products of metabolism lower the pH of the medium. This effect is readily observed by incorporating the indicator dye phenol red into the medium *Phenol red is red at pH 7 4 to 7.8 It* becomes salmon and finally yellow as the pH drops to below 7.0 Conversely, cell necrosis induced by the virus, leaves the medium red, for the dying cultures fail to reach the degree of acidity exhibited by the control cultures. The test can thus be read by color change alone rather than by the microscopic presence or absence of cellular degeneration

The first practicable color test was described by Salk, Youngner, and Ward[37] The modification[39] described below includes the introduction of (1) inexpensive disposable white styrene panels, (2) the use of "stabilized" passage cell suspensions, obtained from monkey kidney cultures by treatment with a chelating agent (versene), (3) the sub-

1 16, 1 64, 1 256, and 1.1,024 dilutions Both these arrangements require a total of 24 cups (6 per panel) for the examination of each serum Each group of 4 panels provides facilities for testing 16 sera

When the serum dilutions have been distributed to a quartet of panels, 0.8 ml of the paraffin oil is added to each cup, and a foil cover is placed on each If desired, the serum-filled panels may be stored a week in the refrigerator, or even longer in the freezer Refrigerated panels must be brought to room temperature to decrease the viscosity of the oil before addition of materials is attempted If the test is completed on the day the serum dilutions are made, the oil may be placed in each cup after the virus or cells have been added

The virus challenges, 100 TCD_{50} in 0 2 ml for each cup, are added to the serum dilutions in the panels, only 1 virus being used in a panel The panels reserved for serum toxicity control receive a blank of 0 2 ml of diluent per cup These additions are followed by an incubation period of 1 hour at room temperature, when 0 2 ml of cell suspension is added, the panels re-covered with foil, and placed in a 37° C incubator for 5 or 6 days

c Additional controls Along with the tests for possible toxicity of individual sera, 3 additional types of control are needed

(1) Each virus titration is done to include a range of dilution sufficient to demonstrate the end point, from one at which all cultures become infected to one in which none is infected Thus, if 100 TCD_{50} are anticipated in 0 2 ml of the virus suspension when at 10^4 dilution, the titration is extended, stepwise, to include the 10^7 dilution, which is 10-fold beyond that in which the 50 per cent end point is expected For $\log_{10}$ intervals, 8 cups are used at each dilution, for 0.5 $\log_{10}$ intervals, 4 cups suffice at each dilution

Virus stocks prepared in tissue culture are stored frozen at −20° C in aliquots of 1 0 ml in stoppered 13 by 100 mm culture tubes For each test, a separate aliquot is thawed Stocks are not refrozen If any lot of virus shows a consistent fall in titer on repeated titrations, it is discarded, for the accumulation of noninfectious virus tends to bind antibody, thus lowering serum titers

(2) Each test must include a reference serum for each antibody type Human serum pools, type-specific monkey hyperimmune sera, and commercial gamma globulin have been useful Reference sera are tested at 2-fold dilutions, using 4 cups per dilution, and 50 per cent end-point titers are calculated for each type

The storage of aliquot tubes of reference sera is recommended Human sera keep satisfactorily at −20° C at 1 4 and 1 8 dilutions for more than 6 months Reconstituted, lyophilized monkey hyperimmune serum can similarly be stored at 1 100 dilution

(3) A cell titration is performed at the beginning and at the end of the test At each time 2 ml of the suspension are diluted with 4 ml of nutrient These 1 3 dilutions, equivalent to the final cell concentration used in virus-serum mixture, are then delivered in 0 6 ml portions to a row of several cups A 2d and 3d row are similarly arranged to contain further 2- and 4-fold dilutions, respectively, of these 1 3 dilutions The cups are covered with paraffin oil and foil and the panel placed in the incubator with the others At the time of reading, about 5 days later, the 1st row should be a bright yellow, the 2d row, a yellow-salmon, and the 3d row, red

in a 1 l Roux bottle and maintained for 5 to 9 days at 37° C. By this time a luxuriant epithelial cell sheet should form and the pH of the medium drop to 7.0 or below To harvest the cells the nutrient is first thoroughly drained from the bottle Then 25 ml of versene solution at 37° C. is added, the bottle restoppered and returned to the incubator If the bottle has not cooled appreciably, in 20 minutes the cells may be loosened from the glass by 2 quick shakes of the bottle in a horizontal plane and then poured into a storage bottle The harvested cells, numbering approximately 20 million per bottle, are readied for storage at 4° C. by adding 75 ml of the nutrient per harvested bottle The addition of nutrient acts to bind any excess of chelating agent, and centrifugation is unnecessary.*

Calculation for further dilution of the cell suspension is based on the following procedure *1 ml of the cell suspension is added to and gently mixed with 2 ml* of the crystal violet solution, and the cells are counted in a hemocytometer. Versene-treated cells do not clump to the same degree as those from primary trypsinized culture If the cell suspension is not immediately used, the counting is deferred to some 24 hours before its anticipated use A sterility test and titration in advance of the test should also be performed The suspension is titrated at several 2-fold dilutions, using 1 0 ml of each dilution in each of 4 stoppered tubes, 13 by 100 mm An index to the condition of the cells is provided by three sorts of observations (1) the color of the tube cultures—not strictly comparable to a cell titration control performed in a panel because in this instance the stopper traps all the CO_2 produced, (2) the morphology of the cells†—especially using granulation as an index of morbidity, and (3) the condition of the culture as a whole, particularly whether a true sheet or islets of cells are present After freshly harvested cells are incubated 16 hours at 37° C, microscopic examination should show complete sheets with a seeding concentration of 135,000 to 180,000 cells per ml, and large islets down to concentrations as low as 90,000 cells per ml Concentrations upward of 135,000 cells per ml will cause enough acid to form to bring about a shift from red to yellow In general 20,000 cells per cup are used within 3 days of their harvest, and up to 30,000 if the suspension is stored from 7 to 10 days

b Test sequence A convenient volume, 2 0 ml, of the lowest serum dilution to be tested is heated 30 minutes at 56° C Two-tenths ml aliquots of each serum dilution are placed in the correspondingly located cups of 4 panels, according to a planned protocol Four equivalent panels are so obtained, 1 of which serves as a *control of serum toxicity*, and the other 3 to be used for challenge of the sera with Types 1, 2, and 3 poliovirus

Two serum dilution schedules have proved especially useful The 1st, for screening, uses duplicate cups for the testing of sera at 3 dilutions, to wit, 1 10, 1 50, and 1 250 The 2d, greater in range, uses a single cup to test sera at 1 4, 1 8,

show the necessary controls Part 4 shows the results on acute and convalescent sera of a patient infected with Type 1 poliomyelitis virus. The pH readings were made on the 5th day of incubation.

TABLE 4

PROTOCOL OF COLOR TEST USING VERSENATED CELL SUSPENSIONS

Part 1 Cell Titration

Cell concentrations	*Color in cup no* 1	2	3	4
4 cups containing 20,000 cells	Y*	Y	Y	Y
4 cups containing 10,000 cells	S	S	S	S
4 cups containing 5,000 cells	R	R	R	R

* Y=Yellow, S=Salmon, R=Red

Part 2 Virus Titration

Virus dilutions	*Type 1*	*Type 2*	*Type 3*
$10^{-4.0}$	4/4†	4/4	4/4
$10^{-4.5}$	4/4	4/4	4/4
$10^{-5.0}$	4/4	4/4	4/4
$10^{-5.5}$	4/4	4/4	4/4
$10^{-6.0}$	3/4	3/4	4/4
$10^{-6.5}$	1/4	0/4	1/4
$10^{-7.0}$	0/4	0/4	0/4

$$\dagger\ 4/4 = \frac{\text{Number of cups showing virus effect (red)}}{\text{Number of cups used}}$$

Part 3 Standard Immune Serum Control Known Type Monkey Immune Serum Plus Test Dose of Virus of Same Type Plus Cells Only Data for Type 1 Illustrated Here

Serum dilutions		*Color in cup no* 1	2	3	4
0 2 ml 1 25	serum + 0.2 ml diluent + 0.2 ml cells (control)	Y	Y	Y	Y
0.2 ml 1 25	serum + 0.2 ml virus + 0.2 ml. cells	Y	Y	Y	Y
0 2 ml 1 100	serum + 0.2 ml virus + 0.2 ml cells	Y	Y	Y	Y
0.2 ml 1 200	serum + 0.2 ml virus + 0.2 ml. cells	Y	Y	Y	Y
0 2 ml 1 400	serum + 0.2 ml virus + 0.2 ml cells	Y	Y	Y	Y
0.2 ml 1 800	serum + 0.2 ml. virus + 0.2 ml cells	Y	S	R	R
0 2 ml 1 1,600	serum + 0.2 ml virus + 0.2 ml cells	R	R	R	R

3 *Reading and interpretation* (Fig 8) The results are recorded on protocol sheets bearing the imprint of 4 ruled lattice patterns, each representing the arrangement of cups in a panel. The shift in color in the cups in which virus is absent or neutralized is expected on the 5th day, but with cells of lessened viability, a few more days may be needed While end points will not change with extended incubation, when a panel is repeatedly inspected during the incubation period it can sometimes be observed that a cup which gives a hint of change—say to a salmon tint—will, a day later, have reverted to red This seemingly false start toward yellow and subsequent return to a shade close to the original red is probably a reflection of the carbon dioxide passage through the oil The gradual, and tardy, release of trapped carbon dioxide from cultures occurs when the amount of virus is insufficient to destroy the cells until after the virus has multiplied appreciably Thus there is enough time in such cultures for cells to metabolize sufficiently to produce a color shift

If the amount of cells used is excessive, enough nonvolatile acid may accumulate to shift the color from red to yellow before the virus has multiplied sufficiently to kill the cells, and a false interpretation might ensue When this is suspected (as it might be when an entire virus-challenged panel turns yellow), it is possible to check on the hypothesis by adding base to each cup in order to change the color from a clear yellow to light salmon The panels are then returned to the incubator for another 1 to 2 days, when those cups with living cells will have again turned yellow, while the light salmon color will be retained by those cups whose cells were already dead when the base was added One drop of 0 05N NaOH from a nonbeveled 19-gauge needle is sufficient to achieve a proper color difference, more may kill cells

Colors intermediate to red and yellow are sometimes encountered in cultures that have reached their end-point reactions The dilution of a toxic factor is easily recognizable when a series of color changes from red to yellow is observed as one progresses to the higher dilutions of serum The corresponding phenomena, in a virus-challenged panel, may be illustrated by the following observations·

Six cups containing serum dilutions of 1 4, 1 8, 1 16, 1 64, 1 256, and 1 1,024 showed the colors red, red, salmon, yellow, yellow, and red, respectively This series indicates that the neutralizing power of the serum is masked at the lower dilutions by some toxic material, whose lessening concentration with further dilution permits cell growth The renewal of cell inhibition on reaching the 1 1,024 dilution is attributed to the insufficiency of neutralizing antibody at that dilution The end point is recorded as the highest dilution of serum that neutralizes the challenge dose· in this example it is that contained in the yellow-colored culture (at 1 256) and this is regarded as the end point The control panel of this serum showed the colors of red, red, *salmon, yellow, yellow, and yellow*

room temperature or in the refrigerator

A typical protocol of a color test is given in Table 4. Parts 1 to 3

they are best seen against a white background; they appear as round, uncolored areas contrasting with the red color of the surrounding living cells, stained with neutral red. At this time, the diameter of the plaques varies from 1 to 3 mm. During subsequent incubation, the plaques increase progressively in size until they become confluent On a given plate, the number of plaques approximately doubles between the 1st and 4th days

2 *Plaque formation in bottle cultures* Hsiung and Melnick[53] have reported on plaque formation in stoppered, flat "prescription" bottles instead of in unsealed petri dishes, eliminating the need for the humidified incubator and CO_2-air mixture In this system, in contrast to petri dishes, monkey kidney cells under agar are stable for more than 12 days Thus plaques may be obtained of certain Coxsackie and enteric cytopathogenic human orphan (ECHO) viruses that have a delayed cytopathogenicity, often requiring 5 to 12 days to become visible

Bottle cultures (4 oz) of trypsinized monkey kidney cells are prepared, using a seed of 10 ml of cell suspension (300,000 cells per ml) The growth medium, consisting of 0 5 per cent lactalbumin hydrolysate and 2 per cent calf serum in Hanks' solution is replaced in 4 to 5 days when an epithelial sheet has grown out and the pH has fallen to below 7 0 Just before use, the nutrient fluid is removed, the virus sample in 0.3 ml is introduced, and the culture incubated at 37° C for 1 hour Then approximately 10 ml of melted agar (1 5%) in Earle's salt solution, containing 2 per cent calf or monkey serum and 0.0017 per cent neutral red, are added to each bottle to cover the cell sheet When the agar solidifies, the bottles are turned over and incubated at 37° C

Plaques in bottles have been obtained with the 3 types of poliovirus, with 6 types of Coxsackie virus (A9, B1-5), and with 9 types of ECHO viruses[54] The sensitivity of the plaque method in petri-dish and bottle cultures was the same for the polioviruses, however, the plaque method was found to be more sensitive, ranging from 2 to 10 times in different experiments, than the tube method using the microscopic cytopathic end point

Plaques of Coxsackie virus A9 (Grigg strain) and those of the polioviruses were indistinguishable both macroscopically and microscopically Coxsackie virus Types B2 (Ohio-1 strain) and B5 (Kentucky strain) also produced circular plaques but with diffuse boundaries Types B1 (Conn-5 strain), B3 (Nancy strain), and B4 (Texas 13 strain) produce plaques earlier than the B2 and B5 types, and their plaques more closely resemble those of poliomyelitis

With the ECHO viruses, plaque formation was much delayed as compared to the 2 to 3 days required for the poliovirus plaques to

Part 4 pH Neutralization Test with Sera of Patient Infected with Type 1 Poliomyelitis Virus

Serum dilutions	*Type 1 virus**		*Type 2**		*Type 3**	
	Acute	*Convalescent*	*Acute*	*Convalescent*	*Acute*	*Convalescent*
1 4	Y	Y	Y	Y	R	R
1 8	Y	Y	Y	Y	R	R
1 16	R	Y	Y	Y	R	R
1 32	R	Y	Y	Y	R	R
1 64	R	Y	R	R	R	R
1 128	R	Y	R	R	R	R
1 256	R	S	R	R	R	R
1 512	R	R	R	R	R	R
1 1,024	R	R	R	R	R	R

* 100 TCD_{50} of each virus used in test Control cups containing *serum* dilutions without virus were *all yellow*

Part 5 Results of Serum Titers

Type 1 specific monkey *serum*		1 800
Patient's serum, Type 1, acute		1 8
	convalescent	1·256
Type 2, acute		1 32
	convalescent	1 32
Type 3, acute		<1 4
	convalescent	<1 4

G. Virus-Induced Plaques

Using trypsinized cells, Dulbecco and Vogt have developed a technic for producing virus-induced plaques, in which each virus particle produces one plaque [26] HeLa and *amnion* cells may also be used

Cultures are prepared in 60 mm. diameter petri dishes as described above After a continuous cell *layer* has formed, the culture is washed 2 to 3 times *with* phosphate-buffered saline (PBS). The washing fluid is removed, and 0 3 ml of virus in *PBS is added* The plates are incubated at 37° C for 30 minutes to permit the virus particles to be adsorbed onto the cells The infected *cultures* are covered with 3 ml. of melted agar and held at room temperature for 10 minutes to allow the agar to solidify. They are then replaced in the incubator supplied with the CO_2 mixture

1 *Characteristics of plaque formation* In an infected culture, both the aspect and the number of virus-induced plaques vary with the time of incubation. Small plaques may sometimes be seen microscopically as soon as 24 hours after infection *In oblique* light, against a dark background, the plaques stand out as small, brilliant areas *After* 48 hours,

3 *Plaque formation using cell suspensions instead of monolayer cultures*[26] The principle of this technic is similar to that used in bacteriophage work. 4 ml. of nutrient-agar medium are poured into empty 60 mm. petri dishes and allowed to harden (bottom agar layer) The plates may be used at once or stored in the refrigerator, in case of storage, they should be held at 37° C. for 2 hours before use A cell suspension, 0.35 ml. in volume but containing about 3 times the quantity of cells required to seed a monolayer culture, is diluted into 0 7 ml of melted nutrient agar medium to give a final agar concentration of 0 6 per cent, 0 1 ml of the virus at the appropriate dilution is added, and the entire contents of the tube poured onto a plate containing a bottom agar layer Dulbecco and Vogt[26] point out the advantages of this technic (*a*) cell suspensions can be used immediately without waiting for the formation of monolayer sheets, (*b*) the method can also be used for cells that are difficult to obtain in monolayer cultures or that will retract under agar, (*c*) the plating efficiency is approximately twice that of monolayer cultures both for free virus and for infected cells One disadvantage of the method is that the minimum cell concentration used as inoculum is more critical than in monolayer cultures since cells suspended in agar degenerate more easily if the cell concentration is below a certain level The formation of plaques with cell suspensions has been obtained with monkey kidney cells and HeLa cells infected with poliomyelitis virus and with chick embryo fibroblasts and L cells infected with vesicular stomatitis virus

4 *Spot-testing technics* Dulbecco and Vogt[26] have introduced spot-testing for the scoring of viral characters, such as heat resistance or serologic type For the latter, for example, monolayer cultures are overlaid with nutrient agar containing type-specific antiserum A loopful of the virus suspension to be tested is deposited on the agar and incubated If appropriate dilutions of virus are used, spots of degenerated cells will develop on plates containing the heterotypic antiserum, whereas they will be absent from plates with homotypic antiserum The same technic has also been applied for cells suspended in agar [26] Agar cell suspensions were, furthermore, successfully used for replicate plating, as developed by Lederberg for bacteriophage studies Plates on which plaques have developed are printed onto velveteen and replicated onto plates containing cells suspended in agar (containing, for example, specific antiserum)

VI HELA HUMAN EPITHELIAL CELL CULTURES

Several workers have attempted to use strains of cells which could be cultivated in vitro as host cells for poliomyelitis, in the same way that certain lines of bacterial cells act as host cells for bacteriophage The most successful application has been achieved by Syverton and his colleagues, who found that Gey's HeLa strain of human epithelial cells, originally derived from carcinomatous tissue of the cervix, supports the growth of poliomyelitis virus.[8] The cells can be transferred in suspension to yield, on glass, sufficient growth in 5 to 10 days for cultivation

become clearly visible ECHO virus plaques were first obtained only after 5 or 6 days of incubation, and these increased in size but not much in number after the 7th to 8th day Occasional strains, however, required 12 to 13 days for these plaques to become manifest. ECHO virus plaques were more irregular in shape than those seen with the polioviruses (Fig. 9). The boundaries of the plaques were always diffuse, and, microscopically, healthy cells could be seen within the areas of degeneration Many attempts to produce ECHO virus plaques in petri dishes met with failure, undoubtedly because the cultures deteriorated in the petri dishes before the beginning of plaque formation could be seen.

The plaque method in bottle cultures has also been applied to the direct isolation of pure lines of viruses from human stools. Plaques of poliovirus types 1, 2, and 3 and a number of ECHO viruses of different types were obtained from fecal materials in which viruses had previously been isolated and identified By selecting, for passage, virus colonies from plaques appearing after different incubation periods, and with different morphologic patterns, 2 enteric viruses (belonging to the polio and ECHO groups) have been isolated directly as pure lines from a single fecal specimen [53] Heretofore, the presence of 2 cytopathogenic agents in the same specimen has often led to confusion until the judicious use of specific antisera was able to unravel the situation

Agar overlay When ready to use, mix A and B below

A *Nutrient medium*

Earle's saline (10 × concentrated without phenol red and $NaHCO_3$)	18.0 ml
Sterile distilled water	60.0 ml
Calf (or monkey) serum	3.6 ml
$NaHCO_3$, 7.5%	5.4 ml
Neutral red (1:1,000, sterilized at 15 pounds for 15 minutes)	3.0 ml
Total	90.0 ml
Add Penicillin	18,000 units
Streptomycin	18,000 μg

For use, bring to 37° C

B. *Agar*

Noble agar (Difco)	2.7 gm
Distilled water	90.0 ml

Melt agar, then sterilize at 15 pounds for 15 minutes, allow to cool to 43° C before use

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of the virus. The cytopathogenic response of the cells to the virus is rapid, taking place in 12 to 72 hours, and yielding virus in high titer. Cells, which grow in sheets without plasma on a glass surface, may be suspended by treatment with 0.5 per cent trypsin, at pH 7.4 to 7.6, at 37° C. for 60 to 90 minutes Direct cellular counts of suspensions may be made in a hemocytometer to permit the dispensing of replicate cultures to ordinary test tubes. The cells settle and stick to the wall of the test tube during incubation in a slightly inclined position. Recent studies have demonstrated the value of the HeLa strain for the isolation of virus and for the measurement of neutralizing antibodies.

The following sections on HeLa cultures have been largely supplied by Dr. Jerome T Syverton and Dr. William F. Scherer, of the University of Minnesota. Further details on the use of these cultures are given in a recent report from their laboratory.[6]

A. Media for Growth and Maintenance of Cells

The medium to initiate cellular growth consists of 50 per cent human adult serum, 2 per cent chick embryonic extract, and 48 per cent balanced salt solution at pH 7 4 Because of poliomyelitis antibodies in human sera, this component must be removed before the virus is added

The medium for cellular maintenance consists of 90 per cent maintenance solution and 10 per cent chicken serum. The maintenance solution is a modification by Hanks and Scherer of Anfinsen's solution It is described in the Appendix. Penicillin and streptomycin are employed routinely in all media for mass cultivation of cells and for viral cultivation at levels of 50 units per ml and 50 μg. per ml, respectively.

Serum pools from 2 to 5 human donors are made to avoid the use of serum deficient in antitryptic effect The serum pool is used directly for making media, or stored at 4° C until needed The final 2.5 ml of serum withdrawn from the 250 ml centrifuge tube is used in bacteriological tests for sterility by transfer in duplicate of 0.5 ml (a) to two rabbits' blood agar slants, (b) to two Sabouraud's agar slants, and (c) to two tubes of thioglycollate liquid medium for incubation for 7 days at 37° C and 20° C, respectively

In a series of classical experiments, Eagle[30] has determined most of the specific nutrients that are essential for the growth and multiplication of HeLa cells. However, a medium containing 13 amino acids and 7 vitamins, each at optimum concentration and supplemented with glucose, did not permit growth unless 5 to 10 per cent human serum was added (The medium is described in the Appendix.) The HeLa strain has recently been adapted to growth in a medium in which the human serum has been replaced by horse serum.[43] HeLa cells adapted

to growth in horse serum are as susceptible to the cytopathogenic effect of the polioviruses, and are as efficient in producing virus, as HeLa cells grown in human serum [57]

B. Cellular Cultivation

Strain HeLa cells are cultivated in a complete serum-containing medium, on a glass substrate to provide surface for cellular multiplication They are cultured for the production of cells in quantity in 200 ml square, screw cap bottles, or in larger flat-sided bottles, and for test purposes in 16 mm diameter test tubes stoppered by screw caps or by rubber stoppers * The cultural vessels are incubated at 36° C in a stationary position at a slant that permits the fluid to cover fully the cellular growth

Bottles are commonly fed twice weekly, tube cultures seldom require feeding The first feeding consists of the addition of 2 5 ml of human serum to the 8 ml in each 200 ml bottle, or by the addition of 0 25 ml of 80 per cent human serum to each tube When subsequent feedings are indicated, feeding is accomplished by replacement of from half to two-thirds of the old medium with new medium (40% human serum) After 3 to 14 days of cultivation at 36° C, more commonly 7 days for tubes and 10 to 14 days for bottles, the cellular population is fully adequate for the preparation of subcultures as described above, or for the support of viral growth The average cellular population, when used for transfer or for viral cultivation, is 5-8 $\times 10^6$ for bottles and 60,000 to 100,000 for tubes When a cellular culture reaches maximal growth as shown by confluent coverage of the glass surface and evidence of continued proliferation, it is removed from the 36° C incubator More commonly, the cells at this time are used immediately by transfer to bottles for increasing the supply of stock cells, or to tubes for virus experiments However, cells can be maintained satisfactorily for future use by incubation at 30° C and feeding twice weekly Feeding is accomplished by withdrawal of about half of the spent medium and its replacement by an equal quantity of 40 per cent human serum

C Preparation of Cells for Transfer

HeLa cells free in suspension for transfer and other purposes are readily made available by the use of the Rous-Jones trypsin method [29] to release and break up cellular sheets from glass An inexpensive preparation of commercial trypsin, "Bacto-trypsin, 1 250," as a 0 5 per cent suspension in maintenance solution, pH 7 4 to 7 6, exerts satisfactory proteolytic activity without "toxic" effects The trypsin suspension is twice filtered by employing (*a*) ash-free filter paper and (*b*) a Bush type filter under positive pressure The filtrate upon being fast frozen and kept at -20° C provides a uniform enzyme preparation for long periods of time The trypsin preparation employed as routine is added to a cellular culture in a volume of 8 ml for a 200 ml bottle, or 1 0 ml for a 16 mm tube, whereupon the container is incubated at 36° C for 60 to 90 minutes The transfer to a centrifuge tube and the production

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* Pure gum rubber, West Co , Phoenixville, Pa Rubber-lined screw caps are available from the A H Thomas Co , Philadelphia (size 28, Catalog No 2849-B for bottles) and from the Kimble Glass Co , Toledo, O (size 15 for 16 mm tubes)

of a uniform cellular suspension are effected by employing a serologic pipette to mix the suspension thoroughly until it consists of single cells or groups of no more than several cells For the preparation of pools of cells, trypsinized cells from bottles or tubes are mixed before centrifugation After horizontal centrifugation of the cellular suspension for 10 minutes at 1,000 r p m, the supernatant fluid containing the trypsin is discarded and the cells are restored to volume, or greater, by adding the old medium and new complete medium

1 *Cellular enumeration* The cells released by trypsinization and resuspended are enumerated by the transfer with a sterile capillary pipette of an aliquot of the suspension to a hemocytometer The cells should be diluted sufficiently to avoid crowding of cells in the hemocytometer Upon microscopic enumeration, the cellular concentration is adjusted by the addition of fresh complete medium to provide a final 100,000 cells per ml (Medium from bottle cultures after 3 to 4 days of incubation may be used to make up 30 per cent of the new medium to conserve the supply of human serum)

2 *Preservation of cells at subzero temperatures*[45] HeLa cells may be preserved for at least 7 months if 20 to 30 per cent glycerol is added to the suspending medium Better preservation will result from storage at –60° than at –20° C., and from thawing in 1 to 2 minutes than in 30 to 60 minutes After thawing, the cells are removed from the suspending medium by centrifugation at 1,500 to 2,000 r p m for 5 to 10 minutes The cells are resuspended in 40 per cent human serum in Hanks' salt solution

D Preparation of Tube Cultures (Fig. 10)

Cells in suspension in a concentration of 100,000 cells per ml are distributed to test tubes, 0 4 ml per tube, or to bottles, 8 ml per bottle The apparatus employed for dispensing to tubes is basically similar to that devised by Evans and Earle, but it has been simplified and adapted for the preparation of replicate cultures of HeLa cells en masse It consists of an all-glass open cylinder with a delivery tube at the base, a helical stirring rod operated by a 1/18 h p. gear-driven motor with a rheostatic control keeps the cells in suspension A piece of rubber tubing is used to protect the shaft of the stirring rod upon its attachment to the motor. The cylinder is capped by a glass hood, which in turn has over it a second glass hood attached by a rubber tube to the shaft of the stirring rod A third glass hood attached by a rubber tube stopper or by a rubber vacutainer stopper to the dispensing needle helps to prevent contamination The delivery tube of the cylinder is attached to a Cornwall pipetting unit to enable the rapid delivery of 0 4 ml aliquots to test tubes, 16 by 150 mm The tubes are stoppered and placed horizontally immediately to permit the cells to settle out on the glass surface. Cultures are kept stationary at 36° C. during the growth period and for maintenance at 30° C.

E Isolation and Cultivation of Virus

The following procedure (Fig. 11) is recommended as routine for each test material which is prepared according to methods given in the previous chapter on Poliomyelitis.

The tubes with moderately heavy cellular growth (60,000 to 100,000 cells), commonly from 5 to 10 days old, are selected for each sample The nutritive medium is removed from each tube by using a pipette or long needle attached to suction and replaced by 0 9 ml. maintenance solution to wash the cells and wall of the tube Scraping of cells from the glass should be carefully avoided An hour later a second replacement is made with 0 9 ml chicken serum diluted 1 10 in maintenance solution to ensure adequate dilution of any poliomyelitis antibody in the original nutritive medium. Washing the cells twice to remove antibody by dilution obviously is unnecessary when the serum employed for the growth of cells is known to be free of antiviral substances 0 5 ml of the diluted chicken serum is adequate for maintaining normal morphology of about 60,000 to 100,000 cells at 37° C for 4 to 7 days; 1 0 ml of medium usually suffices for about 10 days The test inoculum, 0 1 ml, is added, and the tubes are placed at 36° C Microscopic examination daily reveals the presence of virus by the rapidly progressive cellular destruction that results from infection When cellular destruction occurs, commonly in 12 to 72 hours, the fluid is harvested for typing When the time requisite for the cultivation of virus or other test material exceeds 5 to 7 days, however, the supernatant fluid is replaced by maintenance solution containing 10 per cent chicken serum

1. *Cytopathogenicity* The cytopathogenic effect of poliomyelitis virus is entirely comparable to the sequence of changes that has been described for the infection by poliomyelitis virus of other epithelial cells in tissue culture. The cultures are observed microscopically for cellular changes in 18 to 24 hours after infection, and thereafter daily for not more than 7 days If virus be present, total degeneration is observed in 18 hours to 6 days, commonly in 24 to 72 hours The changes observed are recorded according to a standard scale (Fig 12)

0 = No change

1 + = Early degeneration of epithelial cells. The cells may be rounded and transparent, the cells situated peripherally may be swollen, undergoing disintegration and partially released from the glass Similar changes may occur in cultures from 3 to 7 days' old as the result of nutritional or other disturbance

2 + = Degeneration as evidenced by distortion, swelling, or shrinkage of the cells, total loss of intercellular bridges, eversion, and release from the glass; the nuclei are karyorrhectic or pyknotic

F. Immunologic Identification of Cytopathogenic Strains of Virus

Each unknown strain of cytopathogenic virus is identified as to immunologic type by employing hyperimmune monkey sera representative of each of the three known types of poliomyelitis virus. For each test, 8 tubes containing 60,000 to 100,000 HeLa cells (5 to 10 days old) are selected and washed free of human serum as indicated above. The content of each tube within 30 to 60 minutes after the 2d washing is replaced by maintenance solution containing 10 per cent monkey serum. For control purposes normal monkey serum is placed in tubes 1 and 2, antipoliomyelitis Type 1 serum in tubes 3 and 4, Type 2 antiserum in tubes 5 and 6, and Type 3 antiserum in tubes 7 and 8. The suspension under test for virus is added, 0 1 ml to each tube The tubes are observed microscopically daily. Commonly in the presence of poliomyelitis virus, the cells in 6 of the 8 tubes are destroyed in 1 to 4 days and rarely not until the 5th or 6th day Accordingly, in the absence of cytopathogenic changes, the final reading is not made until the 7th day. The unaltered cells in 2 of the tubes reflect the protective or neutralizing effect of one of the type-specific antisera and thereby indicate the immunologic type of the test strain of virus.

G Neutralizing Antibody Determinations

The principles of the poliomyelitis neutralization test as used with mice or with tissue cultures are described in the chapter on Poliomyelitis and should be consulted. Only a bare outline of the procedure as employed in HeLa cells will be described here (Fig. 13).

After harvest of the cells as already described, they should be suspended to a concentration of 100,000 cells per ml. in medium containing 40 per cent human serum and 60 per cent Hanks' balanced salt solution 0 25 ml. quantities of this suspension (25,000 cells) are dispensed in sterile tubes, stoppered, and incubated in a slanting position at 37° C. They should be fed with 0 25 ml of the serum medium on the 3d day and will be ready for tests on the 6th or 7th day. An alternate technic is to dispense 0 4 ml. of the suspension (40,000 cells) into each tube These will be ready on the 4th day, and feeding is not required. When the tubes are ready for use the human serum is removed and replaced with 1 ml. of maintenance solution containing 10 per cent monkey or chicken serum If the human serum is not screened for poliomyelitis antibodies before its use, the cells should be washed thoroughly, twice with maintenance medium, before the step given above.

The three standard viruses (Types 1, 2, 3) must have been titrated previously in the HeLa cell system by adding 0.1 ml. of serial log dilutions to duplicate tubes. Each daily test must incorporate control titrations with new vials of each virus.

Several dilutions of inactivated serum should be employed. Pairs of acute and convalescent serum must be tested together. The virus-serum mixtures are made by combining equal volumes of 1/4, 1/8, 1/16, 1/64, 1/256, and 1/1024 dilutions of serum with previously titrated virus diluted to provide 100 TCD_{50} in 0.1 ml. of the final mixture. It is suggested that sera with higher end points may be subsequently re-examined at higher dilutions, those which are negative with the lowest dilution may also be re-examined in stronger concentrations.

After standing 1 hour at room temperature, 0.1 ml. of each virus-serum mixture is added to single, or preferably duplicate, tubes of cells and incubated at 37° C. The test is read by direct microscopic examination on the 4th day and discarded. Any evidence of viral action on the cells is to be interpreted as no neutralization and so recorded. Tissue controls should accompany each test to rule out nonviral disintegration of cells. Control tests with dilutions of standard type specific sera and standard virus preparations should be performed with each day's run of neutralization tests. The color test for antibodies in which cell suspensions are added to virus-serum mixtures[37–39] can also be performed with HeLa cell suspensions.[40,41]

H. Other Human Cell Lines

In addition to the HeLa strain, a number of other cell lines have recently been adapted to continuous culture, chiefly following methods developed by Gey.[49] They have proved susceptible to poliomyelitis as well as to other viruses. Chang[46] has carried 4 human epithelial cell lines, from normal conjunctiva, liver, kidney, and appendix. Other epithelial cell strains have been adapted to continuous transfer from human cancers.[47,48,51]

Fibroblast lines from human foreskin and uterus have also been carried in serial culture.[50] Some of the newly developed lines have been grown in synthetic medium fortified by 10 per cent human serum.[30,55] Like HeLa, the cells grow rapidly and are maintained easily on glass surfaces. They are readily released by trypsin, or by mechanical procedures, from the surfaces on which they are growing to allow the preparation of uniform suspensions of cells that can be counted and from which replicate cultures can be made.

Swim and Parker[50] found that strains of cultured cells may be preserved in the refrigerator for periods over 6 weeks Within 2 to 3 days after cultures are placed in the refrigerator, a large proportion of the cells tend to contract, cell processes tend to withdraw, and the cells assume a spherical shape. There are no apparent changes during the next 2 months When cultures are withdrawn from storage, they are placed at 37° without replacing the medium The cells gradually extend their processes and in a few days assume the form characteristic of the strain and begin to multiply. If the culture is to be continued in cold storage, the cells should be subcultured before they are returned to the refrigerator. Some workers recommend that the fluid phase be removed from the cells before the culture is stored in the refrigerator.

In addition to the stable cell lines described before, human amnion cells provide a readily available source of large amounts of normal human cells that can be grown in primary cultures.[52] Human placentae and membranes can be obtained wherever deliveries are made. Such cells have yielded as much virus as that obtained in monkey kidney cells

The recommended procedure is as follows·[52]

The membranes are cut from the placenta and dropped into a phosphate-buffer solution at pH 7 2 containing penicillin and streptomycin The amnion is stripped from the chorion and is washed repeatedly in fresh changes of phosphate-buffer solution. The tissue may be cut into pieces approximately 2 cm. square and stirred gently in a 0.25 per cent trypsin-buffer solution The liberated cells are decanted at 20-minute intervals, centrifuged at low speed, washed twice, and diluted with the culture medium

The cells of primary and secondary cultures grow equally well in media containing homologous or heterologous serums· cord, human, horse, ox, or lamb. At a level of 20 per cent, the serum yields better growth than at a level of 40 per cent The medium for large-scale culturing consists of 20 per cent ox serum in either 199 or Earle's balanced salt solution containing 0 5 per cent lactalbumin hydrolysate (see Appendix for preparation of these media) Embryo extract gives no additional response and is omitted

The culture cells were uniformly found to be epithelial, with two groups of cell sizes. The smaller cells were more numerous, and each flattened to cover an area of approximately 4 to 10 μ^2 The cytoplasm appeared homogeneous, with few granules or vacuoles The larger cells, which varied in number from preparation to preparation, each covered an area of 20 to 60 μ^2 They assumed bizarre forms and had prominent, parallel fibrous structures in their cytoplasm

In both the large and small cells poliovirus induced marked cytologic changes The amount of virus produced by the cultures, measured as plaque-forming units on monkey kidney plates, was the same order of magnitude as is usually obtained from cultures of monkey kidney cells The plaque titer obtained on amnion cells, however, was 3 to 6 times higher than on monkey kidney cells[56]

VII. APPENDIX

A Preparation of Materials

1. *Type-specific antiserum.* Monkeys are hyperimmunized according to the procedure incorporating the Freund adjuvants[31,32] Antigen is tissue culture fluid having a high titer, at least 10^6 TCD_{50} per ml, incorporated into an emulsion with the adjuvant mixture (9 parts Bayol F and 1 part Arlacel A) The emulsion may be conveniently prepared in a Waring blendor, or it may be prepared by repeated passage of the mixture through a needle attached to a syringe The emulsion is injected intramuscularly 3 times at 2-week intervals The dose per monkey may range from 1 to 5 ml Monkeys are bled 4 weeks after the 3d injection. A larger supply of serum can be prepared from each monkey if 30 ml of blood is taken on 3 successive weeks A booster dose is then given, and the procedure repeated for several cycles Serum is best stored frozen.

Mountain[58] has described a simple and inexpensive method for producing antisera with a high degree of neutralizing capacity for poliomyelitis viruses By repeated intravenous injection of rabbits with small doses of poliomyelitis viruses grown in tissue culture, antisera with a high degree of homotypic but little or no heterotypic neutralizing capacity have been produced regularly. Such antisera develop also, but to a lesser degree, the capacity to lyse or agglutinate the cells in culture (in the absence of virus)

2 *Hanks' balanced salt solution*[9] All water employed is twice distilled, the second distillation being carried out in a glass still, or water purified by ion exchange resins may be used Salts of analytic reagent grade are employed. Stock solutions A and B are prepared

Solution A Weigh out 160 g NaCl, 8 g KCl, and 4 g $MgSO_4 \cdot 7H_2O$ Dissolve these salts in about 800 ml H_2O Dissolve 2.8 g $CaCl_2$ in about 100 ml H_2O Mix and make up to 1,000 ml with H_2O Add 2 ml chloroform as a preservative, stopper, and store at 5° C

Solution B Weigh out 3 04 g $Na_2HPO_4 \cdot 12H_2O$, 1 2 g KH_2PO_4, and 20 g glucose and dissolve in approximately 800 ml H_2O Add 100 ml 0 4% phenol red solution and make up to 1,000 ml with H_2O Add 2 ml chloroform, stopper, and store at 5° C Hanks' balanced salt solution is prepared by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of H_2O It is then autoclaved at 9 lbs pressure for 10 minutes, or it may be sterilized by filtration This solution is kept at 5° C Immediately before use, 2.5 ml of sterile 1 4% $NaHCO_3$ solution is added to each 100 ml of Hanks' solution The bicarbonate solution is sterilized by autoclaving for 10 minutes at 9 lbs. pressure It may also be prepared by pressure filtration at concentrations up to 7 5 per cent.

3. *Earle's balanced salt solution.*[33]

	g./l.
NaCl	6.80
KCl	0.40
$CaCl_2$	0.20
$MgCl_2 \cdot 6H_2O$	0.17
$NaH_2PO_4 \cdot H_2O$	0.14
$NaHCO_3$	2.20
Glucose	1.00

The solution is stored in the refrigerator in two stocks, one containing NaCl, KCl, NaH_2PO_4, $NaHCO_3$, and glucose at 10 times the indicated concentration, and the second containing $CaCl_2$ and $MgCl_2$ at 20 times the indicated concentration.

4. *Phenol red indicator.*[9] One gram of phenol red is placed in a flask and N/20 NaOH is added slowly and with agitation until the powder is almost dissolved. Dropwise addition of NaOH is then continued until solution is complete, at which time the color is a deep red Sufficient distilled H_2O is added to bring the volume to 250 ml Addition of excess NaOH gives a purple solution that is not satisfactory It is generally used at 20 mg of dye per l of final medium.

5. *Bovine amniotic fluid.*[14] Fluid is withdrawn from the amniotic sac in the following manner. The gravid uterus is removed *in toto* at the abattoir and conveyed to the laboratory within 2 to 3 hours. It is suspended from a support by a cord. After cleansing and drying the entire surface of the organ, an appropriate site near the embryo and close to the dependent area is selected and lightly cauterized with a free flame To avoid piercing the embryo, a special trochar (bore 1.5 mm, length 22.5 cm) is then carefully passed through this part of the uterine wall into the amniotic cavity. The clear fluid, which usually runs rapidly and freely, is collected in sterile flasks The yield of fluid varies from about 0.5 to 1.5 l., depending upon the size of the embryo. Occasionally, fluids are contaminated with blood, presumably because of the injury to the embryo by the trochar These are not used for the preparation of culture medium Embryos about 7.5 to 25 cm in length are selected After withdrawal of the fluid the embryo is removed for the preparation of embryonic extracts (see below) To the fluid withdrawn from each [illegible]

48 hours to determine the presence or absence of bacteria insensitive to

these antibiotics In the meantime, the fluids from each embryo are kept at about 6° C. in tightly stoppered flasks A pool is then made of the fluids from several embryos Sufficient stock phenol red solution (0.4%) is added to the pooled fluid to yield a final concentration of 0 002 per cent. The material is stored in the icebox. On storage the pH of certain batches was found to attain a high level (e g., 8) This is reduced to 7 5 to 7.6 by "gassing" with a mixture of 5 per cent CO_2 in air just before use.

6. *Beef embryo extract.*[13] Embryos of about 7 5 to 25 cm are obtained as indicated above Muscle and connective tissues are dissected from the bones, and together with the brain, heart, lungs, and kidney are minced for about 30 seconds in a Waring blendor. An equal quantity of balanced salt solution containing 100 units of penicillin and 100 μg of streptomycin per ml is then added, and the mixture again minced for 30 seconds. The suspension is incubated at 37° C. for 30 minutes in a water bath and then centrifuged at 2,000 r p m for 30 minutes in a horizontal head of an International No 2 centrifuge The supernatant fluid is removed, a portion cultured, and the remainder stored in stoppered test tubes at –15 to –20° C until used A precipitate may develop upon storage, but this can be removed by centrifugation before the extract is added to the medium

7. *Chick embryo extract*[6] This is prepared from 9-day embryos by Earle's syringe method modified by the use of a single stainless steel screen. Embryos are removed with sterile forceps and placed in petri dishes From 5 to 15 embryos are transferred to the barrel of a 20 or 30 ml syringe, at the bottom of which is a circular piece of stainless steel mesh, No. 28. The plunger is put in the barrel and the embryos forced through the screen and tip of the syringe into a 50 ml centrifuge tube. Extraction is performed by the addition of one volume of balanced salt solution to an equal volume of tissue pulp, followed by thorough mixing and centrifugation at 2,500 r p m. for 20 minutes The supernatant 1·1, or 50 per cent extract, is stored in convenient aliquots at –20° C Prior to use, the 50 per cent extract is thawed, centrifuged at 2,500 r p m for 20 minutes, and only the supernatant fluid employed Microbiological sterility tests are carried out routinely as described for human serum

An alternative method[44] uses embryos incubated 14 days at 38.5° C The embryo paste obtained by homogenization in a Waring blendor for 1.5 minutes is frozen and then allowed to thaw slowly in the refrigerator. An equal volume of

Earle's balanced saline lacking sodium bicarbonate is added, the pH adjusted to 7 0 to 7.4 with sodium hydroxide, and the mixture allowed to stand for 24 hours with occasional mixing The larger tissue fragments are removed by centrifugation at 1,000 × g for 45 minutes Three mg of hyaluronidase (Worthington, 200-250 TRU/mg) are added per 100 ml of pooled supernate, and the solution is incubated at 37° C for 3 hours The hyaluronidase-treated extract is then centrifuged at 35,000 × g for 1 hour (Spinco preparative centrifuge). The supernate is filtered through a Selas filter of 03 porosity and stored at –20° C.

8. *Chicken plasma and serum.*[6] Blood, 30 ml, is withdrawn from the wing vein of 1- to 2-day fasting roosters, through a No 20 needle into a syringe. The syringe and needle are prepared before sterilization by rinsing in 4 per cent mineral oil in ether to provide a thin film of mineral oil on the inner surfaces The blood is transferred in 10 ml. aliquots to screw-cap tubes that contain 0.2 per cent phenol red, 0.1 ml, and 20 mg % heparin, 0 1 ml Each tube is immediately capped, inverted to mix the contents, and centrifuged for 20 minutes at 2,500 r p m. The plasma is withdrawn from the tubes through a long needle into a 20 to 30 ml syringe to provide aliquots of 10 ml. Chicken embryonic extract, 0 1 to 0.5 ml., (to clot the plasma) and several ml. of air are drawn into each syringe. Usually after several minutes at room temperature, the plasma has clotted The needle is removed from the syringe, and the clot is broken by its forceful expulsion through the tip of the syringe into a test tube. Centrifugation, as above, is repeated, and the serum transferred to tubes for storage at 4° C. Plasma is also stored at this temperature.

9 *Lactalbumin hydrolysate medium.*[42] This growth medium provides an inexpensive source of amino acids

5 per cent lactalbumin hydrolysate solution

Powdered enzymatic hydrolysate of lactalbumin*	50 gm.
Salt solution (without $NaHCO_3$)	950 ml

Dissolve by autoclaving at 10 lbs pressure for 10 minutes

The solution is stable at 4° If a precipitate forms on standing, it should be removed by light centrifugation

Complete medium

Salt solution (Hanks' or Earle's)	880 ml
Calf serum	20 ml
5 per cent lactalbumin hydrolysate solution	100 ml

The following antibiotics are incorporated into the medium at the indicated concentrations per ml tetracyclin, 25 μg, streptomycin, 100 μg, penicillin, 100 units; and nystatin, 100 units

* Nutritional Biochemical Corp, Cleveland, Ohio A typical analysis, according to the producer, gives total nitrogen 12 per cent, amino nitrogen 7.3 per cent, ash 3 6 per cent, NaCl 0 9 per cent, and moisture 6 8 per cent

10. *Synthetic medium for monkey kidney cultures (Rappaport's SM-1).*[61]

*Glucose-salt solution**	gms/l
NaCl	8 0
KCl	0 4
$MgSO_4 \cdot 7H_2O$	0 1
$MgCl_2 \cdot 6H_2O$	0 1
Na_2HPO_4	0 03
NaH_2PO_4	0.02
$CaCl_2$	0 42
$NaHCO_3$	0 78
Glucose	20 0

Trace elements†	$M \times 10^{-6}$/l
$ZnSO_4$	3
$Fe(NO_3)_3$	2
$CoCl_2$	0.2
$MnCl_2$	0.5
$CuSO_4$	0 4

Organic supplements‡	mgm/l
l-cysteine • HCl	30
l-isoleucine	60
d-ribose	30

Buffer§

Tris (hydroxymethyl aminomethane) 8×10^{-3} M, at pH 7.6

* Made in 10 × concentrated, combined stock without $CaCl_2$ and $NaHCO_3$ Sterilized by filtration $CaCl_2$ is made at 100 × concentrated, $NaHCO_3$ is made at 100 × concentrated

† Held as separate stocks, 0 05% concentrations Sterilized by filtration

‡ Stocks 100 × concentrated Sterilized by filtration May be held as combined stock, 40 × concentrated in H_2O

§ Conveniently stored as 2 M stock adjusted to pH 7 6 with HCl May be sterilized by autoclaving at 15 pounds for 15 minutes

Amino Acid Supplement for Maintenance Solution [61]*

	mg/l
l-cysteine • HCl	30
l-isoleucine	40
l-histidine • HCl	10
l-arginine • HCl	7
l-lysine • HCl	8
l-methionine	6
l-threonine	14

* Conveniently made from sterile stocks to give 40 × concentrated mixture in H_2O and diluted into salt solution with trace elements and glucose as given above, with 0 9 gms $NaHCO_3$ per l May be buffered with 8×10^{-3} M Tris at pH 7 6

11. *Versene solution* A solution of this chelating agent is used for obtaining suspensions of monkey epithelial cells from kidney cultures grown on glass

	gm/l
Versene	0 2
NaCl	8.0
KCl	0.2
Na_2HPO_4	1 15
KH_2PO_4	0 2

Sterilize by autoclaving

Versene is used as the disodium salt of ethylene-diamine-tetra-acetic acid, obtainable from Versenes, Inc., Framingham, Mass

Earle's balanced saline lacking sodium bicarbonate *is added, the pH adjusted to* 7 0 to 7 4 with sodium hydroxide, and the mixture allowed to stand for 24 hours with occasional mixing The larger tissue fragments are removed by centrifugation at 1,000 × g for 45 minutes Three mg of hyaluronidase (Worthington, 200-250 TRU/mg) are added per 100 ml of pooled supernate, and the solution is incubated *at 37° C for 3 hours The hyaluronidase-treated extract is then centrifuged at* 35,000 × g for 1 hour (Spinco preparative centrifuge) The supernate is filtered through a Selas filter of 03 porosity and stored at -20° C

8. *Chicken plasma and serum* [6] Blood, 30 ml , is withdrawn from the wing vein of 1- to 2-day fasting roosters, through a No. 20 needle into a syringe The syringe and needle are prepared before sterilization by rinsing in 4 per cent mineral oil in ether to provide a thin film of mineral oil on the inner surfaces. The blood is transferred in 10 ml aliquots to screw-cap tubes that contain 0 2 per cent phenol red, 0.1 ml, and 20 mg % heparin, 0.1 ml. Each tube is immediately capped, inverted to mix the contents, and centrifuged for 20 minutes at 2,500 r p m. The plasma is withdrawn from the tubes through a long needle into a 20 to 30 ml syringe to provide aliquots of 10 ml. Chicken embryonic extract, 0 1 to 0 5 ml., (to clot the plasma) and several ml. of air are drawn into each syringe Usually after several minutes at room temperature, the plasma has clotted. The needle is removed from the syringe, and the clot is broken by its forceful expulsion through the tip of the syringe into a test tube. Centrifugation, as above, is repeated, and the serum transferred to tubes for storage at 4° C. Plasma is also stored at this temperature.

9 *Lactalbumin hydrolysate medium.*[42] This growth medium provides an inexpensive source of amino acids

5 per cent lactalbumin hydrolysate solution.

Powdered enzymatic hydrolysate of lactalbumin*	50 gm
Salt solution (without $NaHCO_3$)	950 ml

Dissolve by autoclaving at 10 lbs pressure for 10 minutes

The solution is stable at 4°. If a precipitate forms on standing, it should be removed by light centrifugation

Complete medium.

Salt solution (Hanks' or Earle's)	880 ml
Calf serum	20 ml
	100 ml

indicated

icillin, 100

* Nutritional Biochemical Corp , Cleveland, Ohio A typical analysis, according to the producer, gives total nitrogen *12 per cent,* amino nitrogen *7 3 per cent,* ash *3 6 per cent,* NaCl 0 9 per cent, and moisture 6 8 per cent

To prepare maintenance solution in 100 ml lots

[illegible]

contamination is more likely to occur during mixing

13 *Eagle's medium for growth of HeLa and other human cell lines*[30]

L amino acids*	mM	Vitamins†	gm/ml
Arginine	0 1	Biotin	10^{-6}
Cystine	0 05	Choline	10^{-6}
Glutamine	2 0	Folic acid	10^{-6}
Histidine	0.05	Nicotinamide	10^{-6}
Isoleucine	0 2	Pantothenic acid	10^{-6}
Leucine	0 2	Pyridoxal	10^{-6}
Lysine	0 2	Thiamine	10^{-6}
Methionine	0 05	Riboflavin	10^{-7}
Phenylalanine	0 1		
Threonine	0 2		
Tryptophane	0.02		
Tyrosine	0 1		
Valine	0 2		

* Stored in the refrigerator as a single stock solution containing 20 or 100 times the indicated concentration of each amino acid, except glutamine The glutamine is stored frozen as a 200 mM solution

† Stored frozen as a single stock containing 100 or 1,000 times the indicated concentration of each vitamin

Earle's salt solution containing 0 1 per cent glucose as listed above in this Appendix is used Penicillin and streptomycin are incorporated at 0 005 per cent each, phenol red at 0 0005 per cent

Human serum (10%) is essential for the optimal growth of the cells

The amount of poliovirus released into the medium by HeLa cells was found to be unaffected by the omission of serum proteins, amino acids, or vitamins However, the omission of both glucose and glutamine resulted in a marked decrease in virus production The omission of each singly had no effect or caused only a partial reduction

14 *Medium No 199*[8] Its preparation as revised by Healy and Parker in 1953 follows

a Stock solutions Aqueous solutions are prepared with distilled water passed

12. *Maintenance solution for HeLa cultures*[6]

Preserve stock solutions I, II, III, and IV with $CHCl_3$, 1-2 ml/liter (slight excess)

	Component	For 1000 ml 10 × stock solution		Final conc. (mg/liter)
I	Balanced salt solution (BSS, Hanks') 10 × conc.			
	1 NaCl (biological grade) (a)	80.0 g	Dissolve the BSS salts of blocks *1* and *3* in approximately 400 ml glass-distilled water each and combine. Dissolve the $CaCl_2$ in 200 ml g.d. water and add to the above. Bring to final volume of 1100 ml. If necessary, filter through ash-free filter paper into a glass-stoppered pyrex bottle	8000.0
	KCl (b)	4.0		400.0
	$MgSO_4 \cdot 7H_2O$ (a)	2.0		200.0
	2 $CaCl_2$ (c)	1.4		140.0
	3 $Na_2HPO_4 \cdot 2H_2O$ (a)	0.6		60.0
	KH_2PO_4 (b)	0.6		60.0
II.	$NaHCO_3$ (a) (1.4% in glass-distilled water)			1000.0
III	Glucose (a) (10% in glass-distilled water)			2000.0

	Component	Amount	Dissolve in	Combine	Final conc. (mg/liter)
IV	Amino acids			Combine 500 ml. 10 × stock	
	Parenamine 15% with tryptophane (d)	10 ml	in 100 ml glass-distilled water		300.0
	Glycine	100 mg.			20.0
	Histidine (free base) − DL (e)	100 mg			20.0
	Cystine − L (e)	75 mg			15.0
	Glycerol (a)	2.5 gm	in 100 ml g.d. H_2O		500.0
	Na pyruvate (e)	3.2 gm			640.0
	Na acetate $\cdot 3H_2O$ (c)	5.64 gm			1128.0
	Succinic acid (c)	50 mg	in 100 ml g.d. H_2O		10.0
	1 − malic (e)	25 mg.			5.0
	Adenine $SO_4 \cdot 2H_2O$ (f)	34 mg	in 100 ml g.d. H_2O		6.8
	Guanine $HCl \cdot 2H_2O$ (f)	8 mg			1.6
	Xanthine (e)	5 mg			1.0
	Uracil (f)	5 mg.			1.0
	Thymine (e)	2 mg			0.4
	Cytosine (e)	2 mg			0.4
	KH_2PO_4 (e)	450 mg	in 99 ml g.d. H_2O (100 ml)		90.0
	Thiamine HCl (e)	5 mg			1.0
	Nicotinamide (e)	2 mg			0.4
	Ca-pantothenate (Dextrorotatory) (e)	2 mg			0.4
	Pyridoxal HCl (e)	2 mg			0.4
	Pyridoxamine dihydrochloride (e)	2 mg			0.4
	D-ribose (e)	2 mg			0.4
	Riboflavine (e)	2 mg			0.4
	i-inositol (e)	7 mg			1.4
	Choline Cl (e)	7 mg			1.4
	Biotin (e)	1 mg	in 20 ml g.d. H_2O — 1 ml		0.01
	Folic acid (e)	1 mg			0.01
	PABA (e)	1 mg			0.01
	Phenol red (g) in I, II, and IV				20.00

(a) Merck—Reagent grade
(b) Baker—Analyzed grade
(c) Mallinckrodt—Reagent grade
(d) Winthrop Stearns, Inc.
(e) Nutritional Biochemicals Corp.
(f) Eastman Organic Chemicals
(g) Difco Co.—T. C. grade

to make a final volume of 100 ml The stock is prepared by making a 1:100 dilution of this solution with water

Sol 9—Adenine hydrochloride dihydrate, 51 mg, dissolved in a final volume of 100 ml of water

Sol 10—The following purines and pyrimidines are dissolved in 200 ml of water made alkaline with 2 drops conc ammonium hydroxide; guanine hydrochloride, 10 mg, hypoxanthine, 10 mg, thymine, 10 mg, uracil, 10 mg, and, monosodium xanthine, 11 4 mg

Sol 11—d-Ribose, 100 mg, and d-2-desoxyribose, 100 mg, dissolved in 100 ml of water

Sol 12—5-Adenylic acid, 10 mg, dissolved in 100 ml of water

Sol 13—Sodium adenosinetriphosphate, 260 mg, dissolved in 50 ml of water The stock is prepared from this solution by making a 1 10 dilution with water

Sol 14—Ferric nitrate, $Fe(NO_3)_3 \cdot 9H_2O$, 36 mg, dissolved in 100 ml of water If 1 drop of conc nitric acid is added, hydrolysis during storage is prevented

b *Preparation of one liter of medium* The stock solutions are combined as follows

Sol	1	Amino acids, acetate, glutamine, Earle's solution	500.0 ml
	2	Tyrosine, cystine	20 0 ml
	3	B vitamins	10.0 ml
	4	Vit C, cysteine, glutathione	10 0 ml
	5	Biotin	10 0 ml.
	6	Folic acid	10 0 ml
	7	Vit A, D, and K, cholesterol, Tween 80	10.0 ml
	8	Vit E	10.0 ml
	9	Adenine	10 0 ml
	10	Purines, pyrimidines	6.0 ml
	11	Ribose, desoxyribose	0 5 ml
	12	5-Adenylic acid	2.0 ml
	13	Adenosinetriphosphate	2.0 ml
	14	Ferric nitrate	2.0 ml

The final volume is adjusted to 1 l by the addition of water, and the completed medium is sterilized by filtration and stored in the dark at room temperature Antibiotics (100 units penicillin and 100 μg streptomycin per ml) are added just before use

B Staining of Tissue Cultures

Cultures for histologic staining are either grown directly in ordinary flasks or bottles or on cover slips placed in bottles or tubes. If the first procedure is employed, the cells are removed from the container after fixation, by peeling them off after collodion (celloidin, parlodion) embedding

1. *Fixatives* Cultures are fixed for 15 minutes to 1 hour in either Bouin's or Zenker's fluid, particularly if one is interested in inclusion bodies. They are then stored in 70 per cent alcohol until stained Bouin-fixed cells are transferred directly from the fixative to the 70 per cent alcohol Zenker-fixed cells should be washed with water before transfer to 70 per cent alcohol.

through an ion exchange column or redistilled in an all-pyrex apparatus All stock solutions except Nos 1, 2, and 9 are stored at 4° C, without filtration, for periods not exceeding 30 days Preferably a fresh lot of Solution 1 is made up each time a new batch of medium is prepared If it is desired to store Solution 1, it should be filtered and stored in 500 ml quantities in the refrigerator. Solutions 2 and 9, which tend to form precipitates in the refrigerator, are stored at room temperature

Sol 1—To 400-450 ml *of water stirred continuously and heated to about 80° C* are added the following phenol red, water soluble, 20 mg, l-arginine monohydrochloride, 70 mg, l-histidine monohydrochloride, 20 mg, l-lysine monohydrochloride, 70 mg, dl-tryptophane, 20 mg, dl-phenylalanine, 50 mg, dl-methionine, 30 mg, dl-serine, 50 mg, dl-threonine, 60 mg; dl-leucine, 120 mg; dl-isoleucine, 40 mg, dl-valine, 50 mg, dl-glutamic acid monohydrate, 150 mg, dl-aspartic acid, 60 mg; dl-alanine, 50 mg, l-proline, 40 mg, l-hydroxyproline, 10 mg, glycine, 50 mg; and, sodium acetate, 81 5 mg The solution is cooled to room temperature and 100 mg of l-glutamine are added The free acid is titrated with N NaOH to pH 7 4, with the phenol red that is already present in the solution serving as indicator.

To the cooled solution are now added the ingredients of Earle's balanced salt solution (listed above in this Appendix) Solution 1 is then made up to 500 ml with water

Sol 2—200 mg l-tyrosine and 100 mg l-cystine dissolved with moderate heating in 100 ml of 0 075 N HCl

Sol 3—The following B vitamins are dissolved in 200 ml. (final volume) of water: niacin, 25 mg, niacinamide, 25 mg, pyridoxine hydrochloride, 25 mg; pyridoxal hydrochloride, 25 mg, thiamin hydrochloride, 10 mg, riboflavin, 10 mg; calcium pantothenate, 10 mg, inositol, 50 mg; p-aminobenzoic acid, 50 mg, and choline chloride, 500 mg The stock is prepared by making a 1 50 dilution of this solution with water

Sol 4—The following are dissolved in 100 ml. (final volume) of water ascorbic acid, 50 mg, glutathione, 50 mg, and, cysteine hydrochloride, 100 mg The stock is prepared by making a 1 100 dilution of this solution with water.

Sol 5—10 mg d-biotin are dissolved in approximately 50 ml. of water containing 1 ml N HCl, and the final volume is then adjusted to 100 ml (The HCl *increases the stability on storage) The stock is prepared by making a 1·100 dilution* of this solution with water

Sol 6—10 mg folic acid dissolved in 100 ml of Earle's balanced salt solution, i e, the substances and amounts as listed above, but dissolved in 1 liter of water.

Sol 7—Two alcoholic tinctures are required to prepare this combined stock solution cholesterol 10 mg/ml in 95% ethanol, and, menadione (vit. K) 10 mg/ml in 95% ethanol The following lipid soluble vitamins are added to a 100-ml volumetric flask, calciferol (vit D), 10 mg, dissolved in 1 ml of the tincture of cholesterol; vitamin A, 10 mg, dissolved in another 1-ml portion of the tincture of cholesterol; and tincture of menadione (vit K), 0 1 ml Finally, 10 ml of a 5% aqueous solution of Tween 80 is added to the flask The mixture is then made up to a final volume of 100 ml and warmed to dissolve the cholesterol. The stock is prepared by making a 1·10 dilution of this solution with water

Sol 8—Disodium α-tocopherol phosphate (vit E), 10 mg, dissolved in water

6a [illegible]

6b [illegible]

4071, 1954

7 Stoler, M, and Gey, M K Destruction of a Human Fibrosarcoma Strain (A FI) in Tissue Cultures Following the Growth of Poliomyelitis Viruses *Bull Johns Hopkins Hosp*, 92 385–393, 1953

8 Morgan, J F, Morton, H J, and Parker, R C Nutrition of Animal Cells in Tissue Culture I Initial Studies on a Synthetic Medium *Proc Soc Exper Biol and Med*, 73 1–8, 1950

9 Weller, T H, Enders, J F, Robbins, F C, and Stoddard, M B Studies on the Cultivation of Poliomyelitis Viruses in Tissue Culture I The Propagation of Poliomyelitis Viruses in Suspended Cell Cultures of Various Human Tissues *J Immunol*, 69 645–671, 1952

10 Farrell, L N, Wood, W, Franklin, A E, Shimada, F T, MacMorine, H G, and Rhodes, A J Cultivation of Poliomyelitis Virus in Tissue Culture VI Methods for Quantity Production of Poliomyelitis Viruses in Cultures of Monkey Kidney *Canad J Pub Health*, 44 273–280, 1953

11 Melnick, J L, and Riordan, J T Poliomyelitis Viruses in Tissue Culture IV Protein-Free Nutrient Media in Stationary and Roller-Tube Cultures *Proc Soc Exper Biol and Med*, 81 208–213, 1952

12. Robbins, F C, Enders, J F, and Weller, T H Cytopathogenic Effect of Poliomyelitis Viruses *in Vitro* on Human Embryonic Tissues *Proc. Soc Exper Biol and Med*, 75 370–374, 1950

13 Robbins, F C, Weller, T H, and Enders, J F Studies on the Cultivation of Poliomyelitis Viruses in Tissue Culture II The Propagation of the Poliomyelitis Viruses in Roller-Tube Cultures of Various Human Tissues *J Immunol*, 69 673–694, 1952

14 Enders, J F Bovine Amniotic Fluid as Tissue Culture Medium in Cultivation of Poliomyelitis and Other Viruses *Proc Soc Exper Biol and Med*, 82 100-105, 1953

15 Youngner, J S, Ward, E N, and Salk, J E Studies on Poliomyelitis Viruses in Cultures of Monkey Testicular Tissue II Differences among Strains in Tissue Culture Infectivity with Preliminary Data on the Quantitative Estimation of Virus and Antibody *Am J Hyg*, 55 301–322, 1952

16 Ledinko, N, Riordan, J T, and Melnick, J L Multiplication of Poliomyelitis Viruses in Tissue Cultures of Monkey Testes I Growth Curves of Type 1 (Brunhilde) and Type 2 (Lansing) Strains and Description of a Quantitative Neutralization Test *Am J Hyg* 55 323–338, 1952

17 Syverton, J T, and Scherer, W F Studies on Propagation *in Vitro* of Poliomyelitis Viruses I Viral Multiplication in Tissue Cultures Employing Monkey and Human Testicular Cells *J Exper Med*, 96 355–367, 1952

18 Li, C P, and Schaeffer, M A Simplified Method for Cultivation of Poliomyelitis Virus in Tissue Culture *Science*, 118 107–109, 1953

19 Wood, W, Franklin, A E Clark, E M, Duncan, D, and Rhodes, A J Cultivation of Poliomyelitis Virus in Tissue Culture III Synthetic Medium in Roller-Tube Cultures *Proc Soc Exper Biol and Med*, 81 434–438, 1952

20 Salk, J E Studies in Human Subjects on Active Immunization against Poliomyelitis I A Preliminary Report of Experiments in Progress *J A M A*, 151 1081–1098, 1953

21 Morann, G L, and Melnick, J L Poliomyelitis Virus in Tissue Culture VI Use of Kidney Epithelium Grown on Glass *Proc Soc Exper Biol and Med*, 84 558–563, 1953

22 Svedmyr, A, Enders, J F, and Holloway, A Complement Fixation with the Three Types of Poliomyelitis Viruses Propagated in Tissue Culture *Am J Hyg*, 57 60–70, 1953

2 *Cultures grown on the walls of the containers*[59] Cultures are fixed in the flask or tube, washed and dehydrated in graded alcohols ending in a 1:1 alcohol ether mixture This is replaced by 2 per cent collodion in alcohol-ether, and this in turn by a 6 per cent collodion in alcohol-ether, allowing at least 2 hours in each solution for satisfactory penetration of the collodion into the tissues. The collodion is then poured from the tubes, rotating them at the same time until the collodion has almost dried in a uniformly thin film Cold water is then run into the tube to harden the membrane, and the film is peeled from the glass with the aid of a small spatula If the films are too dry before peeling, they will shrink and be brittle, if not dry enough only the surface film will peel, leaving the cells behind

3 *Handling of peeled films* The cell-containing films may be handled as celloidin sections and placed on glass slides after staining, dehydration, and clearing Clearing is carried out in 95 per cent alcohol and then by oil of origanum and xylene, since collodion is soluble in absolute alcohol

Clearer, brighter stained cells are obtained, however, if the collodion film is removed before staining This is done as follows [60]

a Place the film on a clean glass slide with the surface containing the cells in contact with the glass, and blot with filter paper, pressing hard

b Cover quickly with oil of cloves for clearing (for at least 2 hours).

c Pass through 3 changes of absolute alcohol, at least 15 minutes in each

d Leave in alcohol-ether until the collodion is dissolved

e Transfer the slide with cells attached to 95 per cent alcohol

f Keep in 70 per cent alcohol until stained

g The cultures are then stained, dehydrated in alcohols, including absolute, and cleared in xylene

4 *Staining procedure* For routine hematoxylin and eosin, Harris hematoxylin to which 5 per cent acetic acid was added, and 0 5 per cent eosin in alcohol, have given good results Tissue cultures require solutions slightly more dilute than those used for sections The cultures are dehydrated, cleared in xylene, and mounted in Harleco synthetic resin or Canada balsam, or any other suitable mounting medium

VIII. REFERENCES

1a Weller, T H The Application of Tissue Culture Methods to the Study of Poliomyelitis *New England J Med*, 249 186–195, 1953

1b Melnick, J L Poliomyelitis *Advances in Virus Research*, 1 229–275, 1953

1c Habel, K, and others Biology of Poliomyelitis *Ann New York Acad Sc*, 61 737–1064, 1955.

2 Enders, J F, Weller, T H, and Robbins, F C Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues *Science*, 109 85–87, 1949

3 Melnick, J L Application of Tissue Culture Methods to Epidemiological Studies of Poliomyelitis *Am J Pub Health*, 44 571–580, 1954

4 Hilleman, M R, and Werner, J H Recovery of New Agent from Patients with ... *Biol and Med*, 85 183–188, 1954

5 ... Parrott, R H, and Ward, T G ... Newly Recognized Group of Com- ... *England J Med*, 251 1077–1086, 1954

44 Bryant, J C, Earle, W R, and Peppers, E V The Effect of Ultracentrifugation and Hyaluronidase on the Filtrability of Chick-Embryo Extract for Tissue Culture *J Nat Cancer Inst*, 14 189–225, 1953

45 Scherer, W F, and Hoogasian, A C Preservation at Subzero Temperatures of Mouse Fibroblasts (Strain L) and Human Epithelial Cells (Strain HeLa) *Proc Soc Exper Biol and Med*, 87 480–487, 1954

46 Chang, R S Continuous Subcultivation of Epithelial-like Cells from Normal Human Tissues *Proc Soc Exper Biol and Med*, 87 440–443, 1954

47 M[illegible]

48 St[illegible]

1955

49 Gey, G O, Bang, F B, and Gey, M K Responses of a Variety of Normal and Malignant Cells to Continuous Cultivation, and Some Practical Applications of These Responses to Problems in the Biology of Disease *Ann New York Acad Sc*, 58 976–999, 1954

50 Swim, H E, and Parker, R F Preservation of Cell Cultures at 4° C *Proc Soc Exper Biol and Med*, 89 549–553, 1955

51 Fjelde, A Human Tumor Cells in Tissue Culture *Cancer*, 8 845–851, 1955

52 Zitcer, E M, Fogh, J, Dunnebacke, T H Human Amnion Cells for Large-Scale Production of Polio Virus *Science*, 122 30, 1955

53 Hsiung, G D, and Melnick, J L Plaque Formation with Poliomyelitis, Coxsackie, and Orphan (Echo) Viruses in Bottle Cultures of Monkey Epithelial Cells *Virology*, 1 533–535, 1955

54 Committee on the ECHO Viruses of The National Foundation for Infantile Paralysis Enteric Cytopathogenic Human Orphan (ECHO) Viruses *Science*, 122 1187–1188, 1955

55 Eagle, H Propagation in a Fluid Medium of a Human Epidermoid Carcinoma, Strain KB *Proc Soc Exper Biol and Med*, 89 362–364, 1955

56 Fogh, J, and Lund, R O Plaque Formation of Poliomyelitis Viruses on Human Amnion Cell Cultures *Proc Soc Exper Biol and Med*, 90 80–82, 1955

57 Habel, K, Gregg, N C, and McBride, W D Susceptibility to Poliomyelitis Virus of HeLa Cells Adapted to Growth in Horse Serum Medium *Proc Soc Exper Biol and Med*, 90 87–89, 1955

58 Mountain, I M Production of Type Specific Antisera to Poliomyelitis Viruses in Rabbits *Proc Soc Exper Biol and Med*, 90 271–274, 1955

59 Enders J F, and Peebles, T C Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles *Proc Soc Exper Biol and Med*, 86 277–286, 1954

60 Reissig, M, Personal communication

61 Rappaport, C Monolayer Cultures of Trypsinized Monkey Kidney Cells in Synthetic Medium Application to Poliovirus Synthesis *Proc Soc Exper Biol and Med* 91 464–470, 1956

23 Black, F L, and Melnick, J L The Specificity of the Complement Fixation Test in Poliomyelitis [illegible]

24 R[illegible]

25 K[illegible]
400, 1955

26 Dulbecco, R, and Vogt, M Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses *J Exper Med*, 99 167–182, 1954, and personal communication

27 Youngner, J S Monolayer Tissue Cultures I Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells *Proc Soc Exper Biol and Med*, 85 202–205, 1954

28 Ledinko, N, and Melnick, J L Poliomyelitis Viruses in Tissue Culture V. Reaction of Virus and Antibody Variables of the Quantitative Neutralization Test *Am J Hyg*, 58 223–247, 1953

29 Rous, P, and Jones, F A A Method of Obtaining Suspensions of Living Cells from the Fixed Tissues, and for the Plating out of Individual Cells *J Exper Med*, 23 555, 1916

30 Eagle, H Nutrition Needs of Mammalian Cells in Tissue Culture *Science*, 122 501–504, 1955

31 Ward, R, Rader, D, Lipton, M M, and Freund, J Formation of Neutralizing Antibody in Monkeys Injected with Poliomyelitis Virus and Adjuvants *Proc Soc Exper Biol and Med*, 74 536–539, 1950

32 Salk, J E, Lewis, L J, Youngner, J S, and Bennett, B L The Use of Adjuvants to Facilitate Studies on the Immunologic Classification of Poliomyelitis Viruses *Am J Hyg*, 54 157–173, 1951

33 Earle, W R Production of Malignancy *in Vitro*, Mouse Fibroblast Cultures and Changes Seen in Living Cells *J Nat Cancer Inst*, 4 165–212, 1943

34 Melnick, J L, Ramos-Alvarez, M, Black, F L, Girardi, A J, and Nagaki, D Poliomyelitis Viruses in Tissue Culture VII Experiences with Viral and Serological Diagnostic Procedures *Yale J Biol and Med*, 26 465–485, 1954

35 Rappaport, C Trypsinization of Monkey-Kidney Tissue An Automatic Method for the Preparation of Cell Suspensions *Bull Wld Hlth Org*, 14 147–166, 1956.

36 Melnick, J L, Rappaport, C, Banker, D D, and Bhatt, P N Stabilized Suspensions of Monkey Kidney Cells Suitable for Intercontinental Shipment *Proc Soc Exper Biol and Med*, 88 676–678, 1955

37 Salk, J E, Youngner, J S, and Ward, E N Use of Color Change of Phenol Red as the Indicator in Titrating Poliomyelitis Virus or Its Antibody in a Tissue Culture System *Am J Hyg*, 60 214–230, 1954

38 R[illegible]

39 M[illegible]

40 Robertson, H E, Brunner, K T, and Syverton, J T Propagation *in vitro* of Poliomyelitis Viruses VII pH Change of HeLa Cell Cultures for Assay, *Proc Soc. Exper Biol and Med*, 88 119–122, 1955

41. Lipton, M M, and Steigman, A J A Simplified Colorimetric Test for Poliomyelitis Virus and Antibody *Proc Soc Exper Biol and Med*, 88 114–118, 1955

42 M[illegible]

43 Perry, V. P, Evans, V. J, and Earle, W R Cultivation of Large Cultures of HeLa Cells in Horse Serum *Science*, 121 805, 1955

44 Bryant, J C, Earle, W R, and Peppers, E V The Effect of Ultracentrifugation and Hyaluronidase on the Filtrability of Chick-Embryo Extract for Tissue Culture *J Nat Cancer Inst*, 14 189–225, 1953

45 Scherer, W F., and Hoogasian, A C Preservation at Subzero Temperatures of Mouse Fibroblasts (Strain L) and Human Epithelial Cells (Strain HeLa) *Proc Soc Exper Biol and Med*, 87 480–487, 1954

46 [illegible]

47

48 Stulberg, C S, Berman, L, and Ruddle, F H Detroit 6 Strain of Human Epithelial-Like Cells Virus Susceptibilities *Proc Soc Exper Biol and Med*, 89 438–441, 1955

49 Gey, G O, Bang, F B, and Gey, M K Responses of a Variety of Normal and Malignant Cells to Continuous Cultivation, and Some Practical Applications of These Responses to Problems in the Biology of Disease *Ann New York Acad Sc*, 58 976–999, 1954

50 Swim, H E, and Parker, R F Preservation of Cell Cultures at 4° C *Proc Soc Exper Biol and Med*, 89 549–553, 1955

51 Fjelde, A Human Tumor Cells in Tissue Culture *Cancer*, 8 845–851, 1955

52 Zitcer, E M, Fogh, J, Dunnebacke, T H Human Amnion Cells for Large Scale Production of Polio Virus *Science* 122 30, 1955

53 Hsiung, G D, and Melnick, J L Plaque Formation with Poliomyelitis, Coxsackie, and Orphan (Echo) Viruses in Bottle Cultures of Monkey Epithelial Cells *Virology*, 1 533–535, 1955

54 *Committee on the ECHO Viruses of The National Foundation for Infantile Paralysis* Enteric Cytopathogenic Human Orphan (ECHO) Viruses *Science*, 122 1187–1188, 1955

55 Eagle, H Propagation in a Fluid Medium of a Human Epidermoid Carcinoma, Strain KB *Proc Soc Exper Biol and Med*, 89 362–364, 1955

56 Fogh, J, and Lund, R O Plaque Formation of Poliomyelitis Viruses on Human Amnion Cell Cultures *Proc Soc Exper Biol and Med*, 90 80–82, 1955

57 Habel, K, Gregg N C, and McBride, W D Susceptibility to Poliomyelitis Virus of HeLa Cells Adapted to Growth in Horse Serum Medium *Proc Soc Exper Biol and Med*, 90 87–89, 1955

58 Mountain, I M Production of Type Specific Antisera to Poliomyelitis Viruses in Rabbits *Proc Soc Exper Biol and Med*, 90 271–274, 1955

59 Enders, J F, and Peebles, T C Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles *Proc Soc Exper Biol and Med*, 86 277–286, 1954

60 Reissig M, Personal communication

61 Rappaport C Monolayer Cultures of Trypsinized Monkey Kidney Cells in Synthetic Medium Application to Poliovirus Synthesis *Proc Soc Exper Biol and Med*, 91 464–470, 1956



FIG 1 A Normal fibroblasts in plasma clot growing out from fragment of monkey testicle B Virus effect widespread granulation, shrinkage, and loss of structure of fibroblasts found 3 days after addition of virus Type-specific immune serum prevents virus-induced cellular destruction Photomicrographs of unstained, test tube cultures

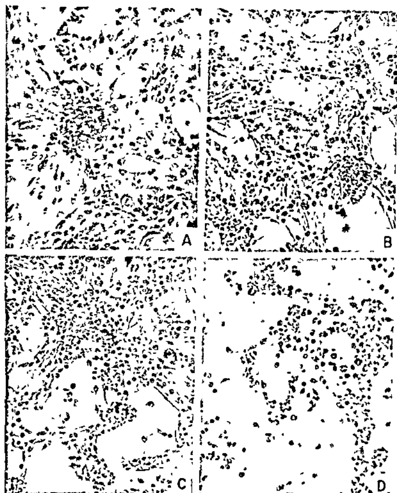


FIG 2. A Normal epithelial cells from monkey kidney growing on the glass wall of a test tube B Early effect (1+) of virus growth a number of cells have become rounded but most of the epithelial sheet does not appear abnormal C Progression of virus-induced degeneration (2+), a larger number of cells are distorted, swollen or shrunken D Advanced stage of virus-induced degeneration (3+) almost no normal cells can be seen, and many of the cells have become detached from the glass surface Photomicrographs of unstained test tube cultures, taken with ordinary microscope

Fig 3 A Phase micrographs of epithelial cells growing on flat glass surface Culture was prepared from monkey kidney fragments which had been treated with trypsin B, C, and D Progressive cellular rounding and degeneration caused by poliovirus Photographs of living cultures taken in phase microscope by Dr Magdalena Reissig

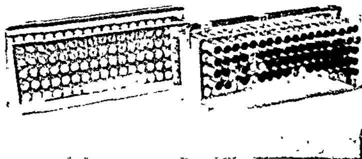
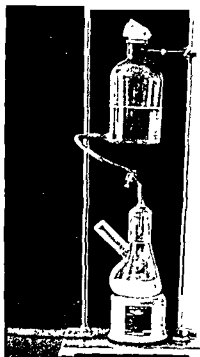


Fig 4 Stationary racks for handling test tube cultures, obtainable from Microbiological Associates, Bethesda, Md Other types of rack are also available ercially



Trypsinization apparatus[35,36]

Fig 5 The sides of a 500 ml, or 250 ml, Erlenmeyer flask have been indented at right angles to the bottom surface in 4 places They should extend about ⅔ the height of the flask and cut into the bottom surface about 2 5 cm This gives the bottom of the flask the shape of a Maltese cross This modification permits satisfactory mixing with cavitation at certain critical volumes Maximum mixing over a wider range of volumes may be obtained if the flask is further modified by accentuating the curvature between the bottom and the sides of the flask This is done by compressing the glass in this region inward and upward The side arm for decanting should be placed so that the tissue tends to be trapped in the bottom when the flask is tilted for decanting The efficiency of trapping may be increased by enlarging the flask just under the side arm

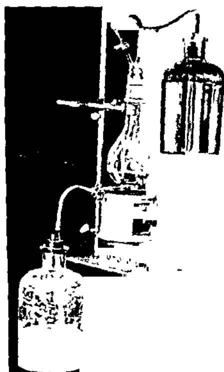


Fig 6 The automatic trypsinizer designed by Dr Catherine Rappaport [37] A 500 ml Kjeldahl flask is modified for use as a mixing chamber Present in the apparatus are a ground glass bacteriologic joint, inlet tube for trypsin from reservoir, an air valve, a flat glass disc, 2 inches in diameter, perforated by 6 bored holes 1 5 mm in diameter, a sloped outlet drain, indentation and modification at sides and bottom of flask to increase efficiency of mixing, and a plastic-covered magnet 40 mm $\times$ 10 mm (diameter) See text for details

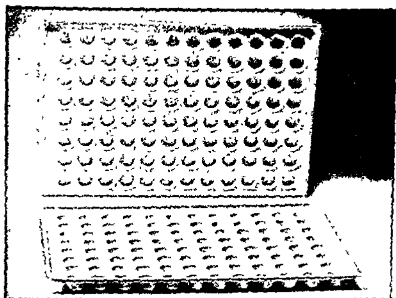


FIG. 7 Disposable styrene plastic panels used for virus and for antibody titrations [39]

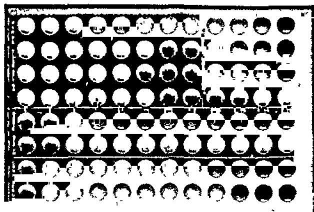


FIG 8 A plastic panel at the time of reading Yellow cups, which photograph light, indicate the presence of live metabolizing cells, red cups, which photograph dark, in the cell titration indicate that too few were added to increase the acidity of the medium significantly Where virus was added, red cups indicate that the cells have been killed

The cell titration in the upper right indicates that the cups receiving 0 2 ml of undiluted cell suspension containing 125,000 cells per ml all metabolized vigorously, as did those receiving one-half this concentration The undiluted cell suspension was used for all the other cups on the panel The virus titration was done using 4 cups per virus dilution, the log of which is indicated at the top of the panel The 50 per cent end point is at $10^{6.0}$ The titration of an acute and convalescent serum from a poliomyelitic patient is shown on the lower half of the panel Two cups were used per dilution of serum The acute serum has a titer of 16, the convalescent, 1,024

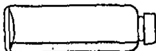


FIG 9 Virus-induced plaques in bottle cultures of monkey kidney cells[53] Circular plaques of poliovirus manifesting well-demarcated boundaries are shown in the bottle on the left Irregularly shaped plaques of ECHO virus, Type 6, with diffuse boundaries, are shown on the right Poliovirus plaques photographed on 6th day of incubation, ECHO virus on 8th day.

→

FIG 10 Syverton and Scherer s illustration of their procedure[6] for the production of strain HeLa cells in continuous culture Cells grown in bottles are placed in suspension by the use of trypsin (or versene) and resuspended in a medium of human adult serum (HAS), 40 per cent, chick embryonic extract (EE), 2 per cent, and Hanks' salt solution (H), 58 per cent The cells are counted in a hemocytometer, adjusted to the required cell concentration, and aliquots of the suspension transferred to bottles and tubes





+
Trypsin

Centrifuge Resuspend cells in $HAS_{40} \pm EE_2H_{58}$



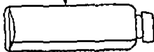


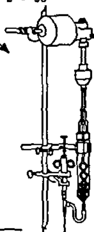
Hemocytometer

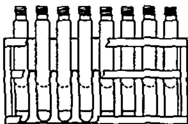
$HAS_{40}BSS_{60}$

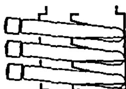
$HAS_{40} \pm EE_2BSS_{58}$











End view

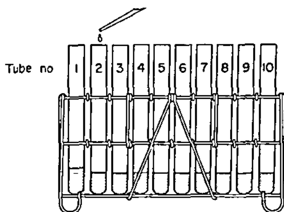










FIG 11 Syverton and Scherer's[6] schematic drawing to illustrate the methodology for virus assay and the results of a test The 1st set of tubes contains diluent to effect dilutions of the test virus suspension within a known range, the 2d set of 5 cellular tube cultures of strain HeLa are for detection of virus, the normal HeLa cells upon infection by virus are destroyed (lower left) or remain unchanged (lower right) in the absence of virus

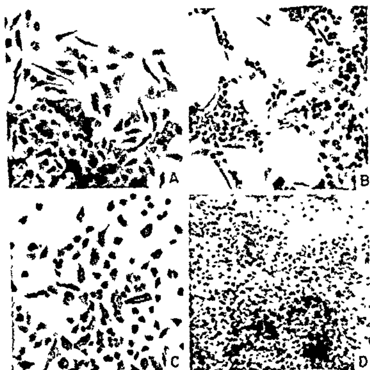


Fig 12 A Normal strain HeLa culture B, C, and D Progressive degeneration as evidenced by cellular distortion, swelling or shrinkage, loss of intracellular bridges, release from the glass, the nuclei become karyorrhetic or pyknotic Photographs of stained preparations kindly supplied by Dr Jerome T Syverton

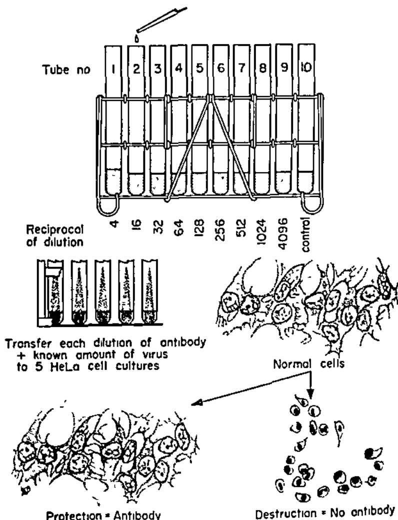


FIG 13 Syverton and Scherer's[6] schematic drawing comparable with Figure 11 to illustrate the procedures and results of the technic employed for quantitation of antibody or other virus inhibitor The 1st set of tubes with known amount of diluent permits dilution of the test unknown serum within a known range for mixture with a constant amount of virus, the 2d set of tubes illustrates the 5 cellular cultures employed to test each successive decrement of antiserum for its ability to neutralize the virus in mixture, the drawings of cells depict the change from normal cells at the start to dead cells (lower right) when free virus persists, that is, unneutralized virus, or to the persistence of unaltered cells (lower left) to illustrate protection from the cytopathogenic effect of free virus

THE COXSACKIE VIRUSES

GILBERT DALLDORF, M.D.

and

GRACE M. SICKLES, B A.
Division of Laboratories and Research
New York State Department of Health
Albany

I. INTRODUCTION

THE COXSACKIE viruses are a group of small viruses which readily infect and cause disease in suckling mice but ordinarily do not cause signs of disease in adult mice [1-4] They are similar to poliomyelitis viruses in their seasonal and geographic distribution, size, resistance to physical agents, and occurrence in the throat washings and feces of infected individuals The nature of the experimental disease permits their classification into two groups[5,6] each of which includes a number of serologic types.[7,8] The Group A strains have commonly been associated with brief, febrile illnesses most common in young children during the late summer months. These illnesses have been further characterized by headache, at

times by stiffness of the neck or muscle soreness, and by the occurrence of small serous blisters on the pharynx (herpangina).[10,11] The Group B strains are believed to be responsible for outbreaks of epidemic pleurodynia or Bornholm disease, the clinical features of which are sudden fever and stabbing pain in the ribs or an extremity (devil's grip), at times with aseptic meningitis.[12-16] One large epidemic of mild, acute meningoencephalitis seems to have been caused by a Group B Coxsackie virus,[17] and a small outbreak of acute fulminating myocarditis in infants has recently been associated with a Group B virus infection.[18]

Coxsackie virus infection is most readily determined by isolation of the agent through the inoculation of suckling mice, followed by neutralization tests with paired samples of the patient's serum and the homologous strain Rise in antibody titer for the homologous type in the serum of infected patients frequently occurs too early to be of much value in diagnosis, and the neutralizing activity for a number of the serologic types present in the serum of many healthy persons is a further limitation to the value of serologic tests alone in diagnosis [11,19-22] Sometimes infection occurs simultaneously with infection with a poliomyelitis virus or a second type of Coxsackie virus. The Coxsackie viruses may be readily distinguished from poliomyelitis virus by the susceptibility of suckling mice and the resistance of monkeys The recognition of concurrent infection with two types of Coxsackie virus may be more difficult.

Some Coxsackie viruses are cytopathogenic and may be isolated in tissue culture [23-26] Much interest attaches at present to the taxonomic position of those viruses isolated in tissue culture from fecal specimens that are neither pathogenic for monkeys nor for suckling mice.[23,24,27-30]

II METHODS OF ISOLATION OF THE VIRUS

A Fecal Specimens

Feces are most likely to yield Coxsackie viruses, but throat washings are at times infectious,[10,13,31,32] and isolations have been reported from cerebrospinal fluid, blood, and tissues [13,32,43] The fluid of herpangina blisters seems to be exceptionally infectious, which is further evidence of the etiologic role of the Coxsackie viruses in this lesion

Specimens for virus isolation should be collected as early as possible in the course of the disease, promptly frozen, and held at low temperatures until used. Virus persists in a considerable number of fecal specimens for long periods even though not maintained under optimal conditions. The Coxsackie viruses are stable in 50 per cent glycerol,

and swabs plunged into tubes of 50 per cent glycerol solidified with agar have proved a satisfactory method for transporting specimens.

Care should be taken to use sterile receptacles for collection of fecal specimens since virus may otherwise be encountered from contamination in previous use The fecal specimen, approximately 8 gms in amount, should be placed in a sterile, wide-mouthed, screw-capped jar without preservative Containers with narrow necks are dangerous to fill, and it is difficult to withdraw the sample Because of the necessity for freezing, the jar should not be more than half full Insert the jar in a double mailing case To protect the laboratory personnel, place the history form between the inner and outer mailing cases and not inside with the specimen jar Freeze in dry ice Pack with dry ice for transportation On receipt at the laboratory, store in a dry-ice cabinet.

1 *Preparation of the fecal suspension* All specimens are prepared in a draft-free room by two workers, each of whom wears a surgical gown, gauze face mask and hair cover, and rubber gloves If spectacles are not worn, a plastic eye shield is used

The specimens to be tested are removed from the dry-ice cabinet about 3 hours before they are required After thawing at room temperature they are kept at refrigerator temperature (4° to 6° C) until used

With a sterile tongue depressor transfer to a mortar a portion of feces, about 2 gms, weigh Grind with sterile sand or other abrasive Add broth-salt solution (0.85% salt solution + 10% infusion broth) to make approximately a 20 per cent suspension Centrifuge at 3,000 r p m for 10 minutes Take off the supernate Recentrifuge it for 30 minutes at the same speed Remove the supernate and add to it for each ml of suspension 500 units of penicillin and 2 5 mg of streptomycin in 0 05 ml Refrigerate overnight Streak on blood and beef extract agar plates

B Throat Swabs

A special double container outfit is desirable, which consists of 2 absorbent cotton swabs on 16-gauge wire 5⅝ inches long, inserted into No. 4 paraffined corks and stoppered in 15- by 125-mm pyrex tubes and 2 corked tubes of the same size containing sterile buffered 50 per cent glycerol solidified with 1 per cent agar

With a swab rub the oropharynx of the patient Remove the cork of a glycerol-agar tube. Thrust the swab into the glycerol-agar and leave it there. Repeat with the second swab and the second glycerol-agar tube

1. *Preparation of specimens* In a sterile mortar, grind the swab in a small amount of broth-salt solution to each ml. of which has been added 500 units of penicillin and 2.5 mg of streptomycin in 0.05 ml.

C. Inoculation of Infant Mice

The mice should be preferably not more than 1 day old. For subsequent passage of strains inciting signs of disease characteristic of Group B, mice 1 day old or younger should be used. For many types of Group A, mice up to 4 days old are satisfactory. All infant mice are pooled and redistributed in groups of 8 by stratified random sampling[34]

With each specimen inject three groups of 8 infant mice; one subcutaneously (0 03 ml), one intraperitoneally (0 05 ml.), and one intracerebrally (0 02 ml.). Observe daily for 14 days; record the signs of disease. With chloroform sacrifice mice that are ill or paralyzed or show tremors or spasms, and harvest the tissues required.

The incubation period may vary from 2 to approximately 13 days Mice injected with strains of many Group A types usually show signs *of disease in 3 to 5 days; of Group B, in 6 to 9 days. Longer and shorter* incubation periods may be seen with both groups

Because it may be removed with less risk of contamination, the brain has been used as the stock tissue for preserving a strain. The virus titer of Group A strains is much higher in the muscles; therefore legs or carcasses are harvested for other purposes

Secure the mouse on the autopsy board on its ventral surface. Wet with alcohol Remove skin. With aseptic precautions remove the brain to a jar containing 50 per cent glycerol and store at refrigerator temperature (4°–6° C.). Remove legs or carcasses to jar to be stored in dry-ice cabinet. Slit the abdominal wall and open the cranium of one whole, skinned mouse inoculated by each route. Transfer to Bouin fixative for histologic study.

III. IDENTIFICATION AND DIFFERENTIATION OF VIRUS

On isolation the Coxsackie viruses fail to induce evident infection in adult mice inoculated intracerebrally

Weigh the tissue Transfer to a sterile mortar and grind with sterile sand or other abrasive Add broth-salt solution to give the required concentration, usually 10 or 20 per cent Centrifuge for 10 minutes at approximately 2,500 r.p m. Take off the supernate. With the supernate, inoculate intracerebrally (0 03 ml) 10 mice weighing 10 to 12 gms Observe for 30 days As a control of infectivity, inject also a group of infant mice. The infant mice should show signs of disease promptly; the weaned mice remain apparently healthy

A DETERMINATION OF GROUP

Observation of the signs of disease in the infant mice will usually indicate the group in which the strain belongs Group A infected animals develop flaccid paralysis usually without encephalitic signs In Group B infected mice, tremors are common, and there may be generalized spasms or paralysis Dyspnea and cyanosis may sometimes be seen Histologic examinations are made for confirmation[6] Group A strains uniformly induce muscle lesions without damage to the central nervous system, fat tissue, or viscera Group B strains are less consistent Focal muscle lesions are frequently present in intraperitoneally inoculated mice, somewhat less often in intracerebrally inoculated animals Involvement of the central nervous system is found in a large percentage after intracerebral inoculation Lesions of the fat pads are frequently seen The interscapular fat pad is the most convenient sample Necrosis of the acinar tissue of the pancreas is not uncommon. Samples of all of these tissues should be collected from early generations fixed in Bouin's fluid, embedded in paraffin, sectioned, and stained with hematoxylin eosin The full complement of anatomical lesions associated with Group B virus infection may not develop until the strain is adapted to mice The muscle lesions due to Group A infections and the fat lesions due to Group B infections can be identified with certainty in the gross by an experienced observer

The muscle lesion is nonspecific in detail as seen in histologic preparations It consists of internal changes in the muscle fibers leading to lysis or coagulation followed by a hyalin appearance and the rapid proliferation of young muscle cells and a variable degree of leucocytic infiltration All or almost all of the striated muscle of infant mice is involved in Group A infections, and the universal distribution is a hallmark of Group A infection

The cerebral lesion associated with Group B infections is also nonspecific and resembles that seen in several other virus infections in which massive necrosis of the cortical neurones occurs within a day or two The lesion of young fat tissue is specific and has been seen under only one other circumstance; namely, in young rodents infected with strains of poliomyelitis virus and dosed with cortisone In infant mice infected with Group B strains of Coxsackie virus, lesions of poliomyelitis do not occur The changes consist of death of immature fat cells, which coalesce and form clumps that become surrounded by a zone of regeneration. The central fat undergoes condensation and after a few

days is quite opaque Calcium deposits quickly follow. The lesion is peripheral in the lobules of the fat.

Necrosis of acinar epithelium of the pancreas is frequently found in mice infected with Group B strains In the young the cells become intensely acidophilic and desquamate into the lumina They shortly become necrotic and are absorbed The cells of the islands of Langerhans are not affected, and in recovered mice only the islands may remain, surrounded by adipose replacement tissue Identical lesions may be found in the pancreases of adult mice infected with certain Group B strains and may serve to identify such infections in animals that sicken but do not die. The faculty of destroying the pancreas is lost if Group B strains are maintained by brain tissue passage, is preserved if carcass is used as inoculum, and is intensified if pancreas is used.

B Determination of Type

Type differentiation is usually made in this laboratory by the neutralization test method in suckling mice. Other investigators have found the complement fixation test to be a satisfactory procedure[9,35-39] for differentiating types and studying their relationships.

With cytopathogenic strains an adequate neutralization test may be made in tissue cultures

1. *Preparation of sera.*

a. Strains. At this time, 19 types have been assigned numbers in Group A. There are undoubtedly others. Five types have been recognized in Group B (Table 1). Stock preparations of the representative strains are maintained as infected brain in glycerol with a few exceptions.

b. Antigens To prepare a virus pool for immunization, large numbers of infant mice are inoculated and sacrificed when signs of disease are apparent The harvested tissues are stored in a dry-ice cabinet.

Prepare suspensions as described on page 156 and store in a dry-ice cabinet.

c. Procedure Serum is produced in large mice and adult hamsters by the intraperitoneal injection of living virus in suspension of muscle For sera to be used in complement fixation tests the homologous animal *tissue is always used.* Sera from animals immunized with muscle tissue are more potent than those prepared with brain suspension.

The dosage of 10 per cent suspension given in four weekly doses is, for mice, 0.5, 1.0, 1.5, and 2 0 ml.; for hamsters, 1 0, 1 5, 2.0, and 3.0 ml. Bleedings are usually taken 7 to 10 days after the last injection. Sera

TABLE 1

CLASSIFICATION OF THE COXSACKIE VIRUSES

Group	Type	Representative Strains		Group	Type	Representative Strains	
		Name	Number*			Name	Number*
A	1	T. T	48249	A	14	G 14[d]	52113
	2	Fl	49190	*Cont*	15	G 9[d]	52108
	3	J Ol	49191		16	G 10[d]	52109
	4	[a]	50246		17	G 12[d]	52111
	5	G S.	5134		18	G 13[d]	52112
	6	C G.	5011		19	N I H[e] 8663	53157
	7	W P	50140				
	8	C D	5010				
	9	P B	50546	B	1	P. O	49683
	10	N K	50548		2	Ohio (Red)[f]	50207
	11	Belgium 1[b]	52148		3	Nancy[g]	50531
	12	Texas 12[c]	51204		4	J V B	51196
	13	Flores	5359		5	Faulkner[h]	53122

* Type culture collection number, Division of Laboratories and Research, New York State Department of Health

[a] Howitt, B F, and Benefield, U R *Proc Soc Exper Biol and Med*, 73 90–92, 1950

[b] Godenne, Mary-O, and Curnen E C *Acta Paediatrica Belgica*, 8 29–42, 1954

[c] Contreras, G, Barnett, V H, and Melnick, J L *J Immunol*, 69 395–414, 1952

[d] Gear, J H [illegible] Second

[e] Robert J [illegible]

[f] Melnick, J [illegible]

[g] Melnick, J [illegible]

[h] Alex J Steigman, University of Louisville, School of Medicine, Louisville, Kentucky

of high neutralizing titer may also be prepared in monkeys by repeated doses of living virus in mouse or hamster muscle suspension

d Determination of specific activity The serum is tested by the neutralization test with the representative strain of the homologous type

Use as virus the representative strain of the type. With Group A sera, test for titer against both brain and muscle suspensions, 10^{-1} In the preliminary test make 10-fold dilutions beginning with 1 5, usually 3 but varying with the type and the tissue In a second test use 2-fold dilutions between those giving complete and no neutralization in the first test

With Group B sera, test against brain suspension, 10^{-1}, 2-fold dilutions beginning with 1 5, usually 5 or 6

In addition, tests for cross reactivity with the other types are carried out.

2. *Complement fixation test.* The complement fixation test with Coxsackie virus antigens, although unsatisfactory as a diagnostic aid, is useful in the serologic classification of strains, especially as a screening procedure Both the test tube technic[35-39] and plate micromethod[9,38,40] have been found satisfactory.

Antigens, in general, are prepared with muscle-bone suspensions of infected suckling mice, partially purified by repeated freezing and thawing followed by high speed centrifugation. Further treatment for removal of nonspecific activity has included acetone-ether,[34] ether,[39] protamine sulfate,[9 38] protamine sulfate and ether,[41] and potassium urate.[42] Activity may be preserved by storage lyophilized,[40] at –70,[40] –20,[9] and in the refrigerator.[42]

The preliminary fixation period used has been, in general, overnight at 4° C., with 30 minutes at 37° C after the addition of the sensitized cells. In addition to controls of complement activity, necessary controls include normal tissue antigen, normal serum, virus antigen without serum, immune serum without antigen, and immune serum with its specific antigen.

3. *Isolation and identification in tissue culture (Group B).* An in vitro method for the isolation and classification of Group B strains is available in tissue cultures of trypsinized monkey kidney cells

Growth of a Group B, Type 1 strain (Conn. 5) with progressive cytopathogenic effect was obtained by Stulberg, Schapira, and Eidam on cultures of mouse interscapular fat pad tissue[25] and mouse skeletal muscle.[43] Weller and co-workers [26] found that another B-1 strain (De Mole) appeared not to multiply in human embryonic skin muscle or in mature uterine tissue but increased in cultures of human embryonic brain and intestine and mature kidney tissue Cytopathologic effects were noted by Crowell and Syverton on HeLa cells with a B-1 strain (Conn. 5) and three strains of B-3 [44] Steigman[45] observed cytopathologic degeneration in renal epithelial roller cultures with several B-2 and a B-5 strain. Sabin[46] isolated a B-4 strain in monkey kidney tissue culture.

Isolation from fecal suspensions in cultures of trypsinized monkey kidney cells of Group B 1-4 has been made in this laboratory and cytopathologic activity noted in monkey kidney cell cultures inoculated with suckling mouse tissue suspension of a number of Group B strains including Types 1 to 5 Certain Group A strains, especially A9, have also been isolated in tissue cultures [63]

a Glassware Tubes (15 by 100 mm) with white rubber-lined screw caps are used routinely.

To clean, soak for 1 to 2 hours in a 0.3–0 5 per cent solution of a detergent suitable for use with local water Scrub by hand to remove soil. Wash in baskets in a CharLab machine in a 0 3–0 5 per cent solution of detergent for 15 minutes at 200°–212° C. Follow the machine washing by two rinsings in the machine in tap water. Rinse tubes individually on a jet rinser with 10 to 15 changes (2 to 3 min) of hot tap water (160°–180° C.) and finally, three times in distilled water Dry in a hot air oven, cap loosely, place in baskets, wrap in paper, and sterilize by hot air at 170°–175° C. for 60 to 75 minutes.

b. Nutrient fluids The fluid used most frequently is the modification of mixture 199[47] with Hanks' balanced salt mixture described by Youngner[48] Mixture 199 with Earle's salt mixture[47] is also used To the former the sodium bicarbonate is added only when ready to use to bring the pH of the medium to 7 2–7.4. Fifty units per ml each of penicillin and streptomycin are also added just before use For growth of cells the 199 mixture (Hanks' or Earle's) is combined with 2 per cent of normal horse serum previously heated at 56° C for 30 minutes No serum is used in the maintenance fluid in the actual test.

c. Tissue. The preparation of trypsinized monkey kidney cells follows in general the methods of Dulbecco[49] and Youngner.[48] Kidneys of cynomolgus and rhesus monkeys have been used

Anesthetize and exsanguinate monkey Remove kidneys and wash with Dulbecco phosphate buffered salt solution,[49] approximately 10 ml. per kidney. Separate cortical tissue as well as possible and with scissors cut into pieces 4 to 5 mm in size. Wash at least four times with approximately 25 ml of phosphate buffered salt solution per kidney. Add 20 ml.

[illegible]

a 50-ml centrifuge tube The pieces of tissue remain in the blendor. Add fresh trypsin and repeat 7 times. Pool the extractions and centrifuge for 5 minutes at approximately 1,000 r p m Discard supernate Resuspend sediment in 20 volumes of mixture 199 + 2 per cent horse serum. Centrifuge for 3 minutes at approximately 600 r p.m Repeat

the washing and centrifugation twice again. Transfer the cell suspension to graduated 15-ml. centrifuge tubes and run for 3 minutes at approximately 1,000 r p m. Note volume of packed cells. Resuspend with 50 times their volumes of mixture 199 (Hanks') + 2 per cent horse serum Strain through 3 layers of cheesecloth Remove a sample. Dilute 1:10 and make a cell count in a hemocytometer. Adjust with the same medium to a final 600,000–800,000 cells per ml.

d. Tissue cultures. Pipette 0.5 ml. of suspension (300,000–400,000 cells) into each tube. Incubate, stationary, at 35°–36° C. in a horizontal position with the neck of the tube slightly raised Aluminum cake pans (9 by 9 in) may be adapted to carry 16 tubes by supplying two neck rests for the tubes.

Cultures are ready for use in from 4 to 7 days, usually 5 and 6

e Isolation Feces are prepared as for inoculation of mice (p. 155).

Replace the fluid on the cultures with medium 199 (Hanks') without serum. Medium 199 (Earle's) may also be used. Dilute the fecal suspension with an equal amount of the same medium With 0.1 ml. inoculate each of 3 tubes Incubate as above Always include uninoculated tissue cultures as controls.

Cell damage is evident in less than 4 to 7 days with maximum effect in less than 5 to 9 days

As with poliomyelitis virus it is necessary to prove that the observed cell damage is specific and not due to cytotoxicity of the inoculum or to another virus To rule out toxic effects a titration in monkey kidney cell subculture (10^{-1} through 10^{-6}) should be made. If suckling mice one day old or less are available they should be inoculated subcutaneously (0 03 ml.). Failure to induce evident disease in suckling mice does not, however, afford proof that the agent is not a Coxsackie virus

f. Neutralization test A satisfactory neutralization test with the specific antisera may be made with the monkey kidney cell cultures The cytopathogenicity of the virus used in the tests is determined by the inoculation of 3 or more 10-fold dilutions combined with an equal amount of Hanks' solution.[50] Pooled normal sera from the same animal species as the immune sera are included as controls. Hanks' solution is used as diluent for both virus and sera Uninoculated tissue culture tubes are incubated with the test and examined daily as controls of the effect of continued incubation on the cells. In the screening test the normal serum and each Group B antiserum may be used diluted 1:5

with approximately 100 infectious doses of virus, in the confirmatory test serial 2-fold dilutions of the specific serum through 1:80 or 1:160

Replace the fluid on the monkey kidney cell cultures with solution 199 (Hanks' or Earle's)

Mix equal parts of virus dilution and serum dilution and hold at room temperature for 1 hour. With each mixture inoculate 3 monkey kidney cell tubes (0 1 ml each) Incubate, stationary, at 35°–36° C. horizontally as above Examine daily for cell destruction The test may usually be terminated within a week. As in tests with poliomyelitis virus the apparent titer of the serum will vary with the amount of virus used as the test dose and the length of the incubation period

4 *Neutralization test in mice* Brain or muscle virus suspension may be employed For convenience we have used brain suspension in type-determination tests when the infective titer is sufficiently high ($LD_{50} = 0.5 \times 10^{-3}$ or less) It has been found necessary to use muscle tissue suspension for most Group A, Type 1 strains and for Types 7, 9, 11, 13, 15, 16, 17, 18, and 19.

The procedure for the screening test varies with the group With strains isolated in this laboratory, one dose of the virus of unknown type, usually brain suspension, 10^{-1}, is tested with the different sera Group A strains are combined with 2 pools of 5 sera each of the first 10 types Pool 1, Types 1, 4, 6, 7, 9, and Pool 2, Types 2, 3, 5, 8, 10 If the strain is neutralized by one of the serum pools, it is then tested with the individual sera in the pool, usually 1 10, against brain 10^{-1} or muscle 10^{-2} or 10^{-3} If neutralization is not obtained with either pool the individual sera of Types 11 to 19 are tested Sera of Types A-13 and A-16 are low in titer, and undiluted serum must be used in screening

With Group B strains each Group B type serum is tested individually, usually undiluted against brain tissue, 10^{-1}

A similar procedure is used with strains sent from other laboratories, but since they are usually received as muscle suspension a preliminary dilution is made If it is not indicated whether they belong in Group A or B, the Group A screening procedure is followed

When neutralization is obtained with serum of a known type, confirmatory tests are based on the previously determined activity of the serum with the representative strain of the type Usually a 10^{-1} dilution of virus brain or muscle-bone suspension is tested with a series of dilutions of serum of known neutralizing activity No routine series of

dilutions can be suggested since they will vary from type to type, but they should be selected on the basis of the previous titrations of the serum with the representative strain

When no neutralization is obtained, or the pattern does not conform with the previous experience, consideration is given to the question of the presence of more than one infectious agent of the Coxsackie group This is not unusual when the test material is sewage or is contaminated with sewage [9] It is also encountered in the individual specimen.[16,33,51]

A very large number of the Group A strains encountered may be classified in the first ten types Failure to be neutralized by Group A, *pools 1 and 2, is, therefore, considered suggestive of a mixture Before* going on to tests with sera of Types 11 to 19, the following procedure is used *Tissue is harvested from the unprotected mice inoculated with* each pool Each of these, virus + pool 1 and virus + pool 2, is again *put in mice with each pool. If no neutralization is obtained, the virus* is then tested with the other type sera (11–19). Frequently, however, *one strain of a mixture will have been eliminated and the other may be* typed The second strain may then be separated by injecting mice with the original sample combined with serum of the known type

Clues to a Group A–Group B mixture may be found during the microscopic examination as when brain or fat pad lesions are found in mice showing signs of disease typical of Group A Suspicion should also be aroused when occasional spastic mice are noted among others showing typical Group A infection Separation of a Group A–Group B mixture is usually not too difficult since the Group B incubation period *is* often longer and the type of the A-strain will have been indicated by the screening procedures Inoculation of suckling mice with the *original* specimen or tissue combined with type-specific antiserum to the A-strain will *yield the Group B* agent After this has been typed, *reinoculation* into mice of the original material with the B-serum will free the A-strain *It is most important to use mice no more than 1 day old for the B-component* since in older mice many B-strains will not induce signs of disease *In mixtures of two Group A strains complement fixation tests will sometimes* provide an indication of the type of one or more of the strains *In the neutralization test partial neutralization by a serum at a dilution* which, through knowledge from previous tests with the representative strain, should *neutralize completely or a definite lengthening by* an antiserum of the period before infection is evident will indicate the line of attack *with the procedure described above*

Since certain Group A types do share antigenic components, cross reactivity may sometimes suggest a mixture which is not present. In these instances it has been customary to pass the infected tissue mixed with the cross reactive serum several times through mice If a mixture is present the virus in the harvested tissue should be completely refractory to the serum used If there is a shared antigenic component the degree of cross reactivity usually continues similar to the original.

When no neutralization is obtained and there is no evidence of a mixture of strains, serum is prepared with the new strain.

a. Procedure Mice of the Albany standard strain 1 to 3 days old are used, 1 day old for Group B The infant mice are pooled and redistributed, usually in groups of 8, by stratified random sampling. The infectivity of the virus used in the tests is determined by the injection of 3 or more 10-fold dilutions of suspension mixed with an equal amount of broth-salt solution Mixtures of virus and pooled normal sera from the same animal species which provided the immune serum are included as controls The infectivity controls are injected last

When required, LD_{50} is estimated by moving average interpolation[52] or the LD_{50} by the method of Reed and Muench

Mix equal parts of virus suspension and serum and hold at room temperature for 1 hour With each dose inject one group of infant mice, Group A strains, intraperitoneally (0 05 ml), Group B, subcutaneously (0 03 ml) Observe daily for 10 days, with some Group B strains, for 14 days Record the signs of disease Remove mice that are dead and sacrifice with chloroform those that are moribund, paralyzed, or spastic

The types encountered to the present appear to be sharply differentiated. Cross reactivity of varying degree has been noted, but it has always been moderate or slight in comparison with the specific activity Reciprocal cross reactivity of Group A, Types 3 and 8, and of Types 2 and 5 has been seen. The A-2—A-5 cross is minimal when mouse immune serum is used Immune hamster serum of A-5, however, has a marked cross with A-2 virus. A-5 serum also possesses neutralizing activity for A-12 This cross reactivity is more evident with A-5 mouse sera than with hamster immune sera, which suggests that it is due to a different antigenic component from that reacting with A-2. Definite reciprocal cross reactivity of A-11 with A-15, and A-13 with A-18 has also been noted Slight though regular cross reactions may be encountered with A-8 and A-12 and A-8 and A-5, particularly in tests with brain tissue Occasional slight cross reactions of A-5 and A-10 serum with A-3

virus, A-8 serum with A-10 virus, A-7 serum with A-14 virus, A-15 virus in A-17 serum, and A-17 virus in A-11 serum have also been observed. Regular cross reactivity has been taken into account in the composition of the Group A serum pools for the screening tests.

IV. REFERENCES

1 Dalldorf, G, Sickles, G. M, Plager, H, and Gifford, R A Virus Recovered from the Feces of "Poliomyelitis" Patients, Pathogenic for Suckling Mice *J Exper. Med*, 89 567–582, 1949
2 Dalldorf, G The Coxsackie Group of Viruses *Science*, 110 594, 1949.
3 Pappenheimer, A M, Kunz, L J, and Richardson, S. Passage of Coxsackie Virus (Connecticut-5 Strain) in Adult Mice with Production of Pancreatic Disease. *J Exper Med*, 94 45–64, 1951
4. Dalldorf, G, and Gifford, R Adaptation of Group B Coxsackie Virus to Adult Mouse Pancreas *J Exper Med*, 96 491–497, 1952
5 Dalldorf, G The Coxsackie Viruses *Bull New York Acad Med*, 26:329–335, 1950
6 Gifford, R, and Dalldorf, G The Morbid Anatomy of Experimental Coxsackie Virus Infection *Am J Path*, 27 1047–1063, 1951
7 Sickles, G M, and Dalldorf, G Serologic Differences among Strains of the Coxsackie Group of Viruses *Proc Soc Exper Biol and Med*, 72 30–31, 1949
8 Dalldorf, G The Coxsackie Virus Group *Ann New York Acad Sc*, 56 583–586, 1953
9 Contreras, G, Barnett, V H, and Melnick, J L Identification of Coxsackie Viruses by Immunological Methods and Their Classification into 16 Antigenically Distinct Types *J Immunol*, 69 395–414, 1952
10 [illegible]
11 [illegible]
285–289, 1952
12 Curnen, E C, Shaw, E W, and Melnick, J L Disease Resembling Nonparalytic Poliomyelitis Associated with a Virus Pathogenic for Infant Mice *J A M A*, 141 894–901, 1949
13. Findlay, G M, and Howard, E M. Coxsackie Viruses and Bornholm Disease *Brit M J*, 1 1233–1236, 1950
14 Weller, T H, Enders, J F, Buckingham, M, and Finn, J J., Jr The Etiology of Epidemic Pleurodynia A Study of Two Viruses Isolated from a Typical Outbreak *J Immunol*, 65 337–346, 1950
15 Lazarus, A S, Johnston, E A, and Galbraith, J E An Outbreak of Epidemic Pleurodynia, with Special Reference to the Laboratory Diagnosis of Coxsackie Virus Infections *Am J Pub Health*, 42 20–26, 1952
16 Huebner, R J, Risser, J A, Bell, J A, Beeman, E A, Beigelman, P M, and Strong, J C Epidemic Pleurodynia in Texas A Study of 22 Cases *New England J. Med*, 248 267–274, 1953.
17 Stanley, N F, Dorman, D C, and Ponsford, J A Hitherto Undescribed Group of Coxsackie Viruses Associated with an Outbreak of Encephalitis *Australian J Exper Biol and M Sc*, 31 31–40, 1953
[illegible] The South African Institute for Medical Research, Johannesburg *Annual Report*,

21 Kraft, L M, and Melnick, J L Complement Fixation Tests with Homologous and [illegible]

22 [illegible] *munol*, 68 663–672, 1952

23 Robbins, F C, Enders, J F, Weller, T H, and Florentino, G L Studies on the Cultivation of Poliomyelitis Viruses in Tissue Culture V. The Direct Isolation and Serologic Identification of Virus Strains in Tissue Culture from Patients with Nonparalytic and Paralytic Poliomyelitis *Am J Hyg*, 54 286–293, 1951.

24 Riordan, J T, Ledinko, N, and Melnick, J L Multiplication of Poliomyelitis Viruses in Tissue Cultures of Monkey Testes II Direct Isolation and Typing of Strains from Human Stools and Spinal Cords in Roller Tubes *Am J Hyg*, 55 339–346, 1952

25 Stulberg, C. S, Schapira, R, and Eidam, C R Virus Growth in Tissue Culture Fibroblasts II Coxsackie Virus (Group B) in Cultures of Mouse Fat Tissue. *Proc Soc Exper Biol and Med*, 18 642–646, 1952

26 Weller, T H, Robbins, F C, Stoddard, M B, and Florentino, G L Propagation of Coxsackie Viruses in Cultures of Human Tissues *J Immunol*, 71 92–97, 1953.

27 Melnick, J L, Riordan, J T, Curnen, E C, and Macrae, A D Tissue-Culture Pathogenic Agents Other than Poliomyelitis Virus Isolated from Cases of Suspected Poliomyelitis *Fed Proc*, 12 454–455, 1953

28 [illegible]

29 [illegible]

30 Melnick, J L, Riordan, J T, Curnen, E C, and Macrae, A D Tissue-Culture [illegible]

31. Sb[illegible]

32 Howitt, B F Recovery of the Coxsackie Group of Viruses from Human Sources *Proc Soc Exper Biol and Med*, 73 443–448, 1950

33 Johnsson, T Laboratory Infections with Coxsackie Viruses. *Arch ges Virusforsch*, 5 250–263, 1953

34 Wadsworth, A B Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, 3d ed Baltimore, Williams and Wilkins, 1947, p 146

35 Casals, J, Olitsky, P K, and Murphy, L C Hemagglutination and Complement Fixation with Type I and II Albany Strains of Coxsackie Virus *Proc Soc Exper Biol and Med*, 72 636–638, 1949

36 Howitt, B F, and Benefield, U R Use of Complement Fixation in the Differentiation of Strains of Coxsackie Virus *Proc Soc Exper. Biol and Med*, 73 90–92, 1950

37 Manire, G P, Sulkin, S E, and Farmer, T W Complement Fixation with a Coxsackie Virus *Proc Soc Exper Biol and Med*, 73 341–343, 1950

38 Kraft, L M, and Melnick, J L Immunological Reactions of the Coxsackie Viruses II The Complement Fixation Test *J Exper Med*, 92 483–497, 1950

39 Beeman, E A, Huebner, R J, and Cole, R M Studies of Coxsackie Viruses Laboratory Aspects of Group A Viruses *Am J Hyg*, 55 83–107, 1952

40 Lépine, P, Sautter, V, Delpy, J, and Artzet F Technique de la déviation du complément sur plaques dans les maladies à virus Application aux virus du groupe Coxsackie *Ann Inst Pasteur*, 84 684–694, 1953

41 Delpy, J [illegible] affections [illegible]

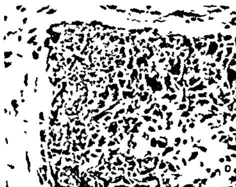
42 Dodd, R. [illegible] Departm[illegible] 1951, p 7.

43 Stulberg, C S, Schapira, R, and Eidam, C R Comparative Characteristics of Two Coxsackie Viruses in Tissue Culture *J Immunol*, 72 107-111, 1954.
44 Crowell, R L, and Syverton, J T. Cytopathogenic Effect of Coxsackie Viruses in Human Epithelial Cell Cultures *Fed Proc*, 13 489, 1954
45 Steigman, A J, School of Medicine, University of Louisville, Louisville 8, Kentucky Personal communication, October 7, 1953
46. Sabin, A B, The Childrens Hospital Research Foundation, Cincinnati, Ohio Personal communication, August 17, 1954
47 Morgan, J F, Morton, H J, and Parker, R C Nutrition of Animal Cells in Tissue Culture I Initial Studies on a Synthetic Medium *Proc Soc Exper. Biol, and Med*, 73 1-8, 1950
48 Youngner, J S Monolayer Tissue Cultures I Preparation and Standardization of Suspensions of Trypsin-dispersed Monkey Kidney Cells *Proc Soc Exper Biol, and Med*, 85 202-205, 1954.
49 Dulbecco, R, and Vogt, M Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses *J Exper Med*, 99 167-182, 1954
50 Youngner, J S, Ward, E N, and Salk, J E Studies on Poliomyelitis Viruses in Cultures of Monkey Testicular Tissue I Propagation of Virus in Roller Tubes *Am J Hyg*, 55 291-300, 1952.
51 Beeman, E A, Parrott, R H, and Cole, R M Simultaneous Occurrence of Two Immunological Types of Group A "Coxsackie" Virus in a Case of Herpangina *Proc Soc Exper Biol and Med*, 78 295-298, 1951
52. Wadsworth, A B Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, 3d ed Baltimore, Williams and Wilkins, 1947, p 151
53 Sickles, G M, Mutterer, M, Feorino, P, and Plager, H Recently Classified Types of Coxsackie Virus, Group A Behavior in Tissue Culture *Proc. Soc Exper. Biol and Med*, 90 529-531, 1955.









Photomicrographs of Coxsackie virus lesions useful in the identification of freshly isolated strains *a* illustrates the extensive muscle lesion that is a hallmark of Group A infection in suckling mice *b* is the early stage of the cerebral lesion that follows Group B infections The cortical neurones are first destroyed The brain is largely destroyed within a short time and then shows widespread necrosis *c* illustrates a very common finding in the early stages of the adaptation of Group B strains to newborn mice *Notice the abnormal fat cells on the surface of the lobule* and compare them with the normal (immature) cells beneath In the histologic preparations changes in coloration help in attracting attention to the early lesions *d* is a somewhat more advanced lesion and shows coalesced, necrotic areas which would soon calcify

ENCEPHALITIS

(Arthropod-borne Virus Encephalitides and Lymphocytic Choriomeningitis)

W. McD. Hammon, M.D., Dr.P.H.

Professor of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania

I. INTRODUCTION

THERE is a large group of neurotropic virus infections of man, encountered in epidemic or endemic form. In temperate climates these occur with sharply delineated seasonal distribution. Their geographical distribution is still poorly delineated. Certain of these can now be grouped as the arthropod-borne virus encephalitides since the viruses have been isolated from either mosquitoes or ticks, and convincing evidence has been presented to indicate that these invertebrate hosts are vectors and occasionally reservoirs.[1] Most of these virus infections are principally of vertebrates (mammalian or avian) other than man, who is a more or less accidental host. On present evidence of a human disease syndrome the following viruses can be assumed to belong to this group: Western equine, Eastern equine, Venezuelan equine, St. Louis, Japanese B, Murray Valley,[2] louping ill, California,[3-5] Russian spring-summer (louping ill?),[6,7] and West Nile[8-13] Probable but less adequately studied members of this group of viruses are Mengo[14,15] and a large number of others isolated either from febrile persons or from mosquitoes in South America, Africa, or the United States[3,16-25b] Lymphocytic choriomeningitis virus, although believed to be spread most frequently by excreta of rodents and to manifest a different epidemiologic and geographic pattern, is capable of experimental arthropod transmission. Since its diagnosis and clinical manifestations follow the general pattern of the others under consideration, it will be included in this discussion

Most of the diseases in this group range in severity from the mildest type of illness, which gives no indication of primary involvement of the central nervous system, through a mild, febrile, meningitic form with spinal fluid changes and other clinical manifestations like those of nonparalytic poliomyelitis, to a severe, central nervous system disorder characterized by stupor, tremors, convulsions, coma, and death.[11,25,26] The more severe cases are those usually recognized. Although these viruses are carried to man by blood-sucking arthropods (which generally acquire infection from the smaller vertebrates[1]), in some infections of man the stage of viremia is fleeting and possibly absent. The same applies to the presence of virus in the spinal fluid. In several types of these infections, only serologic methods are available for laboratory diagnosis unless the disease is fatal, when an opportunity is offered to isolate the virus from the central nervous system tissue. In many of the infections the pathologic lesions are so similar in pattern that the etiology cannot be determined by microscopic study alone. For details of the pathology

of several diseases in the group six papers are recommended.[27-30a] In general, the agent to be sought or the serologic tests to be performed can be judiciously selected[26,31] if one knows what viruses are active in the area where exposure occurred, if the season is that of activity of the recognized vector (spring for ticks, summer for mosquitoes), and if exposure to bites is probable.

As a group, these viruses range in size from 15 to 122 mμ[31a] so are readily filtrable through the more common bacteriologic filters, and they will remain in suspension when centrifuged at speeds from 16,000 to 18,000 r.p.m. in a Spinco No. 30 rotor. Insofar as tests have been made none is adversely affected by the usual bactericidal or bacteriostatic concentrations of penicillin,[32] streptomycin,[32] the sulfonamides,[33] phenyl mercuric borate,[33] or 50 per cent neutral glycerol. Merthiolate should not be used because of its effect on certain viruses.[33a] Thus several procedures are available for rendering suspected tissue suspensions bacteriologically inactive for animal inoculation. The viruses are inactivated by ether[34] and oxidizing agents and stabilized by certain reducing agents.[35,36] The range of pH stability varies considerably from one virus to another.[36-39] All require 10 per cent or more of rabbit serum or other appropriate protein-stabilizing substance to preserve them at constant titer for as long as 2 hours at 37° C. They tend to lose titer slowly over a period of a few days at 5° C. even in this medium. In sealed tubes and in 50 per cent serum at the temperature maintained in dry-ice chests, a concentrated suspension will maintain its infectious titer for many months, and the viruses may be safely preserved thus for years, and possibly indefinitely, without further passage. Preservation up to three months is quite satisfactory at -20 to -30° C. in a mechanical deep freeze and for nine months for some members of the group.[40] These viruses may be lyophilized, and inactivation in the dried state proceeds very slowly even at room temperature. One of the most common, though less effective, means of storage is in 50 per cent neutral glycerol at 5° C.

II. METHODS OF ISOLATION AND IDENTIFICATION OF VIRUSES

A. Precautions and Preparation of Workers

Several laboratory infections have occurred among those working with the Eastern, Western, and Venezuelan equine and with St. Louis, louping ill, Russian Far East, and lymphocytic choriomeningitis viruses.[41] In most of these, the route of infection is not definitely known, but prob-

ably in the majority was by inhalation of finely dispersed virus particles. Under no circumstances should the Waring blendor or other similar mechanical device be used unless under an adequate hood. Previous to vaccination no one should be permitted to work with the Venezuelan or Russian and louping ill viruses themselves or with the inoculated animals. Vaccination[42] is also recommended for those working with Eastern and Western equine virus The three "equine" type vaccines can be obtained from certain manufacturers of biologicals as formalinized chick embryo suspension prepared for horses or prepared especially for human use No vaccine is available for lymphocytic choriomeningitis, but it is strongly recommended that only those known to have a significant titer of "accidentally" or normally acquired antibodies be permitted to work with it, where it is being used, or where infected animals are housed. Accidental infections with clinical manifestations with Japanese B virus have not been reported among the many workers in contact with it, but great precaution should be taken in particular against any type of air dispersal. Smadel's warnings on hazards should be studied by all workers with these agents [42a]

B Sources of Materials

West Nile virus has been isolated from the blood in numerous instances.[9 10 11] In lymphocytic choriomeningitis infection, blood and spinal fluid should be tested for virus These fluids have likewise been repeatedly reported positive in the Venezuelan equine and Russian spring-summer *diseases, but rarely or never with most of the others.* Otherwise, gray matter, preferably selected from the brain stem, midbrain, and hippocampus, is selected for test

C Collection, Shipment, and Storage of Materials

Blood and spinal fluid, if taken for test, should be collected only during the early, febrile phase *Blood should preferably be heparinized,* for virus may be adsorbed to cells.[32] Citrate and oxalate should not be used because they produce convulsions when inoculated intracerebrally into experimental animals All fluids should be collected aseptically. Brain tissue, when possible, should also be obtained aseptically. Virus can most frequently be obtained from patients that have died within the first 5 days *after onset and when autopsy is performed within 2 or 3 hours* after death. Enough isolations, however, have been made under less favorable circumstances to encourage other attempts if no better material is available. Obviously, if embalming is performed prior to autopsy,

the tissues are valueless for the tests to be described. Selected tissues for *pathologic* study should be placed in 10 per cent formalin or Zenker's solution or both.

Immediate inoculation of specimens, though preferable to storage, is not always practical Storage at 5° C to -10° C, obtainable in ordinary electric refrigerators or in a water-tight container surrounded by ice and water, is adequate for 1 or 2 days If longer storage is anticipated the tissue or fluid should be placed in an adequately sealed tube and placed in carbon dioxide ice or other type of storage container maintained at -50° C to -70° C Penetration of carbon dioxide gas has been known to inactivate some of these viruses,[32] so that sealing against entrance of the gas under the greatly reduced pressure of an extremely low temperature is considered important If materials are to be shipped, the same criteria of temperature must be observed Hemolysis of erythrocytes due to freezing does not appear to injure these viruses [32] Frequently, portions of brain tissue placed in 50 per cent neutral, buffered glycerol will yield virus if stored for several weeks at 5° C., and this method may be used to advantage if shipment is essential In no case should materials be left more than a few minutes at or above room temperature

D Preparation of Materials for Inoculation

After bacterial sterility has been demonstrated, or when there is little doubt of it, blood or spinal fluid may be inoculated without further preparation Blood should be tested in several 10-fold dilutions, and also undiluted, since occasionally virus is isolated only after dilution Brain tissue should be suspended in 10 per cent inactivated normal serum (preferably rabbit or other laboratory animal known to be free from antibodies) in saline which contains 625 units of penicillin and 50 μg of streptomycin per ml The suspension can be prepared by thoroughly grinding it for 5 to 10 minutes with a good abrasive such as alundum Either a 10 or 20 per cent tissue suspension is usually prepared After slow speed centrifugation for a few minutes, the supernatant is inoculated The object is to sediment large tissue particles and abrasive Centrifugation at 16,000 to 18,000 r p m for 15 minutes in a refrigerated centrifuge may be required if contamination is excessive

E Selection of Animals

Mice are highly susceptible to all these viruses, and if only one kind of animal is to be used, the mouse is the animal of choice Although any mouse will serve for certain viruses of the group, for satisfactory work with the St Louis virus, the Webster neurotropic virus-susceptible strain of albino mouse (the Swiss-W) is preferred [43] Chick embryos, chicks (less than 6 hours' old), and guinea pigs are equally susceptible or more

susceptible to certain strains of the equine viruses. Guinea pigs are more susceptible to some strains of the virus of lymphocytic choriomeningitis and hamsters to Murray Valley encephalitis virus.[43a] There appears to be no indication to include other animals in the primary attempt to isolate any virus of this group Early work with tissue cultures suggests that with further knowledge certain tissues may be employed advantageously for some of the viruses of this group.

F Route of Inoculation and Age of Animal

Mice, guinea pigs, and hamsters are most susceptible by the intracerebral route For some of the viruses guinea pigs and hamsters develop only an inapparent illness as evidenced by the development of antibodies With well-adapted strains of most of these viruses, the age of the animal has little effect on susceptibility to cerebral injection. This does not apply to certain freshly isolated strains For these strains the younger animals appear to be more susceptible. For virus isolation unweaned mice 3 to 6 days of age or younger are preferred when available A group of 6 or 12 should be inoculated with 0.01 ml intracerebrally and 0 03 ml. intraperitoneally and the suckling mice returned to their mother for nursing. For inoculation a ⅜-inch or ½-inch, 26- or 27-gauge needle is recommended. Unweaned guinea pigs may also be used with advantage, giving 0 05 ml. intracerebrally and 1 0 ml. intraperitoneally If animals are used after weaning, mice aged 3 to 4 weeks may be given 0 03 ml. intracerebrally and 0.3 ml intraperitoneally, and guinea pigs 0 15 ml and up to 5 0 ml, respectively. All intracerebral injections should be made under light ether anesthesia Six or more animals should be used If mice are to be inoculated by the peripheral route only, the age of the animal is very important, for it has been shown[44-47] that an increasing resistance develops in growing animals to infection by this route with most of these viruses. Increased peripheral resistance with age has not been demonstrated for Venezuelan equine, Russian, and some strains of louping ill virus Other routes—intranasal, intracutaneous, or subcutaneous—are not recommended Hamsters are inoculated with 0 1 ml intracerebrally and up to 0.5 ml intraperitoneally.

If eggs are used, they may be infected by any route In general, 6 or more 9- to 12-day-old embryos are inoculated with 0 1 ml by stabbing into the area near but not in the embryo (during visualization), or into the yolk sac, or, if preferred, onto the dropped chorioallantoic membrane After sealing with wax or a paraffin-wax mixture, they are incubated at

35° to 37° C[48] As a whole, when working with this group of viruses, there is rarely any indication for using eggs.

G. Type and Duration of Observation and Behavior of Animals and Eggs

Infant mice must be carefully observed several times a day for signs of illness, for should one become moribund or die it may be promptly eaten by the mother. Other animals are examined twice a day for appearance of signs of infection, and the rectal temperature of guinea pigs should be taken. A clinical, rectal thermometer should be introduced to the 100° F or the 38° C line for a true reading, and only temperatures above 104° F. or 40° C. are of significance Guinea pigs showing an elevation of temperature for 2 successive days without other signs of infection should be sacrificed for passage of brain and spleen (the latter for lymphocytic choriomeningitis), otherwise, they are observed for 20 days unless signs are noted of weakness, tremor, salivation, inco-ordination, convulsions, or paralysis Mice are observed up to 20 days for roughening of the coat, lack of or increased activity, or unusual behavior of any kind—tremors, circling, hunched position, stiffening of the tail, or paralysis The "spin test," consisting of rotating the mouse one way, then the other, while he is suspended by the tail, then watching his reaction after release, is frequently a valuable observation It may induce rolling or clonic or tonic convulsions Any sick mouse is sacrificed for passage of the brain It is not unusual to find only one animal becoming ill during the first passage of material.

Eggs are observed for activity several times a day after the first 12 hours This is done in a dark room over a candler. Death of the embryo, indicated by complete immobility with lack of pulsation and transparency of vessels, occurring more than 12 hours after inoculation, is an indication for passage of the material

H Methods of Passage

Brains or spleens of animals or embryos from eggs are removed aseptically and triturated in a 10 per cent suspension in the same manner as the original tissue was prepared This suspension is cultured and sedimented, and the supernatant fluid is inoculated by the same routes previously employed in a group of the type of animal that has proved susceptible After several successful serial passages the incubation period will usually decrease, inoculation can be performed by the intracerebral route only, and only 2 or 3 animals need be inoculated

A still more sensitive type of test is one employing serial blind passages of inoculated animals or eggs. This method should only be attempted by those with experience and with the full realization of the many possible pitfalls, principally that of picking up a latent agent present in one of the animals.

I. Pathologic Specimens, Smears, Stains, and Filtration

After it is certain that an infectious agent has been transmitted in serial passage, parts of the central nervous system tissue should be examined in stained microscopic sections and by impression smears stained with Giemsa and a stain like Macchiavello's, to rule out larger obligate tissue parasites. Animal inoculation after passage through a Seitz or a Berkefeld V or N filter will establish the filtrability of the agent. Prior to filtration, the 10 per cent suspension in serum-saline should be clarified by centrifugation at moderate speed (3,500 to 15,000 r.p m.).

J. Identification

1. *Animal host range.* At this time it is usually in order to inoculate several species of laboratory animals with the agent, some individuals of each species by the intracerebral route, others by the intraperitoneal or subcutaneous routes. Those inoculated by either the intracerebral or the peripheral routes should receive serial 10-fold dilutions of the virus suspension up to 10^{-5} or 10^{-6}. The results of these trials will frequently give a clue to the identity of the agent. Table 1 is useful for identification of certain viruses of this group. It includes also Theiler's encephalo-

TABLE 1

Comparative Susceptibility of Certain Laboratory Animals to a Group of Neurotropic Viruses (on Primary Isolation or Early Passage)

Virus	Mice, 4 wks or older		Guinea Pigs 350 gms or more		Rabbits		Monkeys	Chick Embryos (Death)	Hamsters	
	i c	i p	i c	i p	i c	i p	i c		i c.	i. p
Western equine	++++	++	++++	++	+++	±	++++	++++	++++	++++
Eastern equine	++++	++	++++	++	+++	±	++++	++++	++++	++++
Venezuelan equine	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++
St. Louis	++++	+	±	0	0	0	±	±	++++	±
Japanese	++++	++	±	0	0	0	++	±	++++	±
West Nile	++++	+++	±	0	0	0	++++	++++	++++	++++
L. C. M	++++	±	++++	+++	0	0	+	+		
Theiler's virus (T.O)	+	0	0	0	0	0	0	0		
Murray Valley	++++	+	0	0	0	0	0	±	++++	++++
California	++++	0	0	0	0	0	0	±	++++	±
Russian louping ill	++++	++++	0	0	0	0	++++	?	++++	++++

myelitis virus of mice. This agent is frequently picked up inadvertently and can usually be recognized fairly well by its limited host range and low titer. It is noted with all these viruses that freshly isolated strains occasionally show peculiar behavior regarding pathogenicity for certain hosts.

Final identification must be made by immunologic tests

2. *Neutralization tests.* Specific hyperimmune sera to the more frequently encountered viruses are essential stock reagents in any virus laboratory. These are most conveniently prepared in rabbits. Injections are given subcutaneously or intraperitoneally, beginning with a small dose, then proceeding with a series of increasing doses given once a week. The rabbit is bled after about 2 or 3 months With certain closely related viruses sera tend to cross more if the rabbit is repeatedly inoculated with large amounts of virus With the Venezuelan equine virus it is wise to begin with formalinized virus, then living virus can be safely used No heterologous serum or other highly antigenic material should be incorporated in the inoculum

Before setting up a neutralization test it is usually most economical to make a preliminary titration of the sample of frozen virus suspension, using 10-fold dilutions Details are given in the section on serologic tests, page 185 The unknown virus is next titrated in the presence of two or more hyperimmune sera to viruses whose properties most closely resemble those of the new virus, and in the presence of a normal serum Table 2 illustrates the primary titration and the setup of the final test

TABLE 2

VIRUS IDENTIFICATION BY NEUTRALIZATION TEST RESULTS OF PRIMARY VIRUS TITRATION AND RECOMMENDED DILUTIONS FOR NEUTRALIZATION TESTS

	Dilution of Virus								
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Primary titration with normal serum	4/4*	4/4	4/4	4/4	4/4 (L)†	3/4	1/4	0/4 (H)†	0/4
Neutralization test proper									
Control serum				+‡	+	+	+	+	
Serum A	+	+	+	+	+	+	+	+	
Serum B	+	+	+	+	+	+	+	+	
Serum C	+	+	+	+	+	+	+	+	

* Numerator indicates number of mice that died, denominator, the number inoculated
† The significance of (L) and (H) is discussed in detail in the test.
‡ + Indicates that this mixture of serum and virus is to be prepared and inoculated.

A series of tubes is set up to receive the serum and the virus dilutions for the neutralization test proper, as shown in the table. The titration with the control serum, included in the test to verify the titer of the virus under the actual conditions of the neutralization test, includes one virus dilution 10-fold more concentrated than the weakest dilution that killed all the mice (L or lowest dilutions) in the preliminary test and each dilution up to and including the dilution which killed none of the mice (H or highest dilution) For each of the hyperimmune sera the lowest dilution used in the series should be three or four 10-fold dilutions stronger than the first in the control series All the dilutions used with the control are also included (L minus 4 or 5 through H). After incubation of these, mice are inoculated with all serum-virus mixtures. At the end of the period of observation for the mice (about 15 days) the LD_{50} for each serum is computed by the method of Reed and Muench[49] (see Table 5). If the specific hyperimmune serum for the unknown virus is included in the test, the LD_{50} will be, depending on the potency of the specific immune serum, two or more dilutions lower than those of the control and other sera In some instances (St. Louis, Japanese B, West Nile, and Murray Valley group) overlapping is expected to occur,[2,50] but neutralization by the homologous serum will usually be greatest

When a hyperimmune serum has been prepared against the newly isolated virus, it is wise to test its protection against several known viruses, the reverse of the test described above.

Identification should not be considered complete by the neutralization test alone, particularly if overlapping antigenicity with any other known virus is recognized. Cross-protection tests are also indicated.

3. *Cross-protection tests.* A series of suitable animals should be vaccinated with a *known* stock strain of the virus selected on the basis of the above neutralization test, and enough normal control animals set aside so that they will be of the same age when the challenge test is performed. These may be mice or guinea pigs, if both animals are susceptible, or only mice if guinea pigs are not susceptible. For immunization, an amount of virus is selected for the first injection that will kill few or none by the intraperitoneal route Or, if the animals are highly susceptible by this route (Table 1), a 10 per cent brain suspension of the virus may be inactivated by a final dilution of 0.2 per cent formalin (U S.P.) and used as such for two inoculations, followed by one or more inoculations of the living virus Injections may be spaced 7 to 14 days apart, and a higher concentration of virus used in each successive inoculation. In most instances 2 weeks after the 2d or 3d inoculation of living virus by the in-

traperitoneal route, the animal will withstand 100 to 1,000 or more LD_{50} doses of the homologous virus by the intracerebral route. A different procedure is required for lymphocytic choriomeningitis virus. Animals should be infected first with an attenuated strain, then with a virulent one, before being used as immunes.

While immunizing the test animals, similar normal ones are used for a preliminary titration to determine the LD_{50} of a frozen ampule of the unknown virus. For this titration animals are inoculated with virus taken directly from the tubes in which the serial dilutions are prepared without further addition of rabbit serum and without incubation A frozen ampule of the virus used for immunization is also titrated The L and H dilutions are determined for each virus

In the test proper (Table 3) the unknown and known viruses are

TABLE 3

CROSS PROTECTION EXPERIMENT AND RESULTS OF PRELIMINARY TITRATIONS AND RECOMMENDED DILUTION FOR CHALLENGE INOCULATIONS

	Dilution of Virus									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Preliminary titrations										
Normal animals with unknown virus	-	-	-	4/4*	4/4(L)†	3/4	2/4	0/4(H)†	0/4	-
Normal animals with Virus A	-	-	-	-	-	4/4	4/4(L)	2/4	1/4	0/4(H)
Test proper										
Animals vaccinated with Virus A, inoc with unknown		+‡	+	+	+(L)	+	+	+(H)		
Animals vaccinated with Virus A, inoc. with Virus A				+	+	+	+(L)	+	+	+(H)
Normal animals with unknown virus				+	+(L)	+	+	+(H)		
Normal animals with Virus A						+	+(L)	+	+	+(H)

* Numerator indicates number of mice that died denominator, the number inoculated
† The significance of L and H is discussed in detail in the text
‡ + indicates that this mixture of serum and virus is to be prepared and inoculated

inoculated into sets of the vaccinated mice, using 10-fold dilutions and including 3 or 4 dilutions more concentrated than L and through and including dilution H. Similarly, the control mice are inoculated with each virus except that only the dilutions representing L minus one through H are used. Vaccinated animals should show *definite protection* in approximately the same degree to each of the viruses (known and unknown) if the identification by neutralization test was correct.

Identification may be further confirmed by vaccination with the unknown virus and challenge inoculation with one or more known viruses. With certain viruses protection against intracerebral inoculation is very difficult to obtain, and in such a case an animal must be used which is susceptible by a peripheral route.[51] If the challenge is to be by intraperitoneal inoculation, vaccination should be by some other peripheral route.

4. *Complement fixation* From some viruses in early passage good complement-fixing antigens may be prepared.[32] This antigen and a normal mouse brain antigen can be tested with a series of known, specific, hyperimmune sera and the virus tentatively identified. The complement fixation test is described on page 197. It has been found, however, that many strains of freshly isolated virus, and occasionally well-adapted strains, do not produce satisfactory complement-fixing antigens, so that this method frequently fails In all instances, a second immunologic test should be employed to confirm results, for related viruses may cross more extensively by complement fixation than by neutralization.

III. SEROLOGIC TESTS FOR IDENTIFICATION OF DISEASE

A. Introduction

Recent work by Casals and Brown,[52,52a] and Clarke and Casals,[53] using the hemagglutination inhibition test, has made it possible to include most of the recognized members of this group of viruses in one of two immunologic groups[54] In group A are Eastern, Western, and Venezuelan equine, Semliki Forest, Sindbis, and Mayaro, in group B are Japanese B, St. Louis, Murray Valley, West Nile, Russian spring-summer, louping ill, Ilheus, Ntaya, Uganda S, Zika, yellow fever, and dengue viruses A few others for which antigens have been made do not appear to belong to either of these two large groups, and antigens have not been successfully prepared against a few others Unfortunately, the hemagglutination tests are so complex and as yet so inadequately evaluated in field practice that at this time they cannot be recommended for the routine diagnostic laboratory The reader is referred to the original publications,[52 53b] and it is hoped that additional data of a practical nature will become available in the near future This type of test will probably serve principally as a screening method to suggest which viruses should be employed in another more specific type of serologic test.

It has been pointed out above that for the group of virus diseases under discussion, serologic tests are of prime importance in arriving at as near a correct diagnosis as is possible. The available methods, however, have several serious disadvantages. Outstanding examples are: (1) diagnoses are usually made in restrospect, after recovery of the patient;

(2) the first of the necessary serum specimens (minimum two) is not always taken during the early acute phase[58c] because the correct diagnosis may not have been considered, and (3) serologic methods are not always sufficiently specific to determine which of certain viruses in group B has been responsible for the infection.

Two types of test are now available for all of the viruses, virus neutralization and complement fixation The first has been used extensively for many years Confidence in its relative specificity, knowledge of many of its shortcomings, and certain partly standardized procedures and modifications have kept it in the position of favor Knowledge, however, regarding the effect of temperature and of storage on certain antibodies necessitated re-evaluation of comparative methods once considered reliable[32,59 63] At the time this is written the final answers to some of these vital questions are as yet undetermined

The complement fixation test is a newer tool in this particular field[64 65] Antigens made by different technics vary greatly in potency and specificity The more specific and sensitive antigens are relatively dangerous to prepare Insofar as both of these commonly used serologic tests are concerned, it does not seem advisable to recommend "standard procedures" Instead, a description will be offered of the tests now performed in one or more laboratories, accompanied by a discussion of some of the known pitfalls and limiting factors

One of the outstanding difficulties encountered in performing or interpreting any of these tests relates to storage and transportation of serum specimens The simpler types of comparative titration tests on paired sera described apply only to sera stored and transported under almost ideal, intelligently planned, and closely supervised conditions Under less ideal, but more frequently encountered, circumstances these tests may be unsatisfactory or misleading, and it is proposed to point out where modifications of each test and certain other considerations may be used to advantage These more varied, less ideal circumstances of collection and storage are those encountered by any state or military special diagnostic laboratory or by a university or other research center which offers its services in the diagnosis of this group of diseases Each one of these laboratories is faced repeatedly and most frequently with the practical problem of how to handle specimens from the individual case The specimens are usually far from ideal in many respects, yet cannot in any way be changed in retrospect The physician wants an etiologic *diagnosis!* The problem in each instance is what test or tests are most suitable for this one peculiar set of circumstances A clinical summary of the case is essential in arriving at this decision At present no single "standard procedure" offers optimal results

B. Collection of Materials

Olitsky and Casals' recommendations[66] are as follows:

Serum It is important to bleed persons for test serum after several hours of fasting (for example, in the morning before breakfast or before meals) and to employ sterile technic About 20 cc of blood is withdrawn, and after it is clotted by keeping it at room temperature for one-half hour, the blood is centrifuged at

2,500 revolutions per minute for twenty minutes Clear serum is then pipetted If a preservative is needed a 1 50,000 dilution of phenyl mercuric borate[33,33a] can be added (or 1·2,500 borate stock, 1 drop to each 1 cc of serum, as an estimate). If a preservative is added the label on the tube should indicate this. The serum is placed in a hard-glass ampule, which is sealed, if a test tube is used, it should be closed with a cork or rubber stopper and the top should be completely waxed. A glass container should be filled only to one-third its volume to prevent bursting during freezing The container is kept at about minus 76° C. by means of carbon dioxide snow until tested It should be sent to the designated laboratory by the fastest possible method, preferably in a vacuum jar holding solidified carbon dioxide If this refrigerant is not available, the serum should be sent fluid, provided it can reach its destination within thirty-six hours

All these precautions are necessary The freezing of serum to maintain its antibody level and to standardize more than one sample for comparative tests from the same source; and obtaining clear, sterile serum to obviate a possible aggregation of virus particles (and thus a nonspecific reduction in titer), which cloudy, fatty or contaminated serum may bring about, and again to standardize more than one specimen from the same person.

The first specimen should be taken as soon as a neurotropic virus infection is suspected, the sooner the better. If taken early enough in infections with certain of the viruses, this sample will fail to neutralize or to give a positive complement fixation reaction, and in such an instance interpretation of a later positive is greatly facilitated. The next specimen may be taken between the 15th and the 21st day A specimen taken 30, preferably 60, days after onset is occasionally essential in the series, especially in St Louis encephalitis and lymphocytic choriomeningitis

C. Neutralization Tests

1. *General discussion.* In general principle, the neutralization test is a method to demonstrate virus-inactivating substances contained in the serum. When a certain quantity of virus is mixed with a "normal" serum and the virus-containing mixture is found to be adequate to kill a laboratory animal, yet when the same quantity of virus mixed with another serum is given to a second animal and death fails to occur, the last-mentioned serum is said to neutralize the virus. In practice several graded amounts of virus or serum are employed, and several animals are inoculated with each mixture; thus crude quantitation is effected.

The neutralization test, in addition to being a diagnostic tool, may show the result of inapparent infections, can be used in epidemiologic surveys, and is used for the partial identification of newly isolated viruses Although the serum of certain species of animals contains nonspecific

antiviral substances, so far as is known neutralization of these arthropod-borne viruses by the serum of man, properly collected and handled, is usually specific, resulting from experience with the particular virus or one antigenically related

The labile factor mentioned above which changes with storage or increased temperature may be one of serious import when using serum that has not been constantly frozen. Shipping a liquid serum during the encephalitis season (hot summer) may result in a marked change in its neutralizing capacity [59,61] Storage of an acute phase serum for 3 or 4 weeks at 5° C. may render it incomparable to a later fresh sample; that is, for test by the ordinary comparatively simple method employing serial dilutions of virus [59,61] Since the labile accessory substance is largely lost by heating at 56° C for 30 minutes,[59,60] or diluted out between 1 5 and 1:10,[61] routine inactivation [60] or employment of serial serum dilutions can be substituted [61] Each of these, however, has its disadvantages The greatest of these is loss of sensitivity in detecting small amounts of antibody. Most laboratories do not routinely employ either of these methods

2. *Animals used and routes of injection* These are essentially the same as described for virus isolation on pages 173 to 174

3 *Types of test.*

a Classified as to whether serum or virus is diluted.

(1) Undiluted serum with serial virus dilutions This is the most commonly applied test, the least beset by technical difficulties, and the easiest to standardize for comparative purposes It cannot, however, be unreservedly approved for the following reasons

(a) It does not represent a true titration of serum antibodies, and results of quantitative comparisons do not always parallel those obtained by serial dilutions of serum [67]

(b) For comparative titrations (essential for diagnosis in most instances), it can be recommended only for use with frozen sera or inactivated sera to which an "accessory substance" has been added This may be furnished by adding fresh or frozen normal human serum known to be devoid of specific antibody for the virus used [32]

(2) Constant amount of virus with serial serum dilutions This test has the advantages of giving a true serum titration, of requiring the least amount of serum of any type of test, and of being applicable to unfrozen sera (accessory factor diluted out), provided end points for all sera

2,500 revolutions per minute for twenty minutes Clear serum is then pipetted If a preservative is needed a 1 50,000 dilution of phenyl mercuric borate[33,33a] can be added (or 1 2,500 borate stock, 1 drop to each 1 cc of serum, as an estimate) If a preservative is added the label on the tube should indicate this The serum is placed in a hard-glass ampule, which is sealed, if a test tube is used, it should be closed with a cork or rubber stopper and the top should be completely waxed A glass container should be filled only to one-third its volume to prevent bursting during freezing The container is kept at about minus 76° C by means of carbon dioxide snow until tested It should be sent to the designated laboratory by the fastest possible method, preferably in a vacuum jar holding solidified carbon dioxide If this refrigerant is not available, the serum should be sent fluid, provided it can reach its destination within thirty-six hours.

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The neutralization test, in addition to being a diagnostic tool, may show the result of inapparent infections, can be used in epidemiologic surveys, and is used for the partial identification of newly isolated viruses. Although the serum of certain species of animals contains nonspecific

trifuged at 2,000 to 3,000 r.p.m. for at least 30 minutes in the cold, or at 3,000 r.p.m. for not more than 10 minutes at room temperature, and the supernatant is then removed to another tube. From this, about 0.8 ml. is placed in each of a number of sterile 1.0 ml. ampules. By use of a 10 ml. syringe and a 2-inch needle the amount may be measured roughly and placed readily in narrow-necked pharmaceutical ampules. These are then sealed, shell-frozen by agitation in a CO_2-alcohol mixture, and stored in a dry-ice chest.

c. Titration of virus. At the same or different times, two or three frozen ampules are titrated. Each titration is made under exactly the same conditions as the neutralization test. An adequate quantity of cold diluting fluid, such as 10-per-cent-inactivated rabbit serum-saline, is first prepared. Since each ampule is titrated separately and in a manner identical to the other, the description of only one is given. Into a set of sterile tubes standing in an ice-water bath, diluting fluid for a series of nine 10-fold dilutions is pipetted accurately. When this has been accomplished, and not before, one ampule of frozen virus is rapidly thawed by agitating in a 37° C. water bath. With a sterile *volumetric* pipette, calibrated to deliver 0.5 ml., this exact amount of virus (0.5 ml. of a 0.5×10^1-fold dilution) * is delivered into the first tube, which should already contain exactly 4.5 ml. of diluting fluid. The pipette is then discarded. This dilution (0.5×10^2-fold) will finally represent a 10^2-fold dilution of mouse brain after an equal amount of the serum to be tested has been mixed with it. With a sterile 1.0 ml. pipette, the contents of tube 1 are mixed and 1.0 ml. delivered into 9.0 ml. of diluting fluid in the next tube. The pipette is discarded. In a similar fashion all dilutions through the 0.5×10^{10}-fold are prepared. Next, six cork-stoppered, short, wide-mouth tubes (from which syringes with ⅜-inch or ½-inch, 26-gauge needles can be easily filled) are selected for serum-virus mixtures. To each, then, is added 0.2 ml. of normal inactivated rabbit serum. Then 0.2 ml. of the 0.5×10^5-fold dilution is placed in the first tube, 0.2 ml. of the 0.5×10^6-fold dilution into the second, and so on through 0.5×10^{10}. A separate pipette is used for each dilution. The final virus dilutions are now 10^5- through 10^{10}-fold. These stoppered tubes are then shaken gently for mixing and placed in a 37° C. water bath for exactly 2 hours, then transferred to an ice-water bath. Six mice

* To avoid the complexity of reciprocals in expressing dilutions, so far as is practical, all dilutions will be stated as so many "fold." Thus a 10^{-1} dilution becomes a 10^1-fold dilution and a 2×10^{-5} dilution is a 0.5×10^5-fold dilution (20%).

to be compared are greater than the 1:10 serum dilution. It possesses the following serious disadvantages, however:

(a) Since only one virus dilution is employed, it is technically difficult to work within the narrow range required. The amount of virus used must not be excessive or inadequate.

(b) The end points of titrations are likely to be more drawn out and irregular than those with undiluted serum.[32]

(c) The test is not readily subject to standardization for reference to any constant or index such as the neutralization index.

(d) This test has been less frequently used and less is known about its variables.

b Classified as to route of inoculation

(1) Intracerebral test The customary route of inoculation for both methods given above is the intracerebral one. This procedure may fail to demonstrate a rise in antibody between two sera of low or moderate titer when a conspicuous difference can be shown by the intraperitoneal route.[51,63] It is, however, the only practical test for most laboratories, because mice which are weaned can be employed, and small age differences in the mice are irrelevant

(2) Intraperitoneal test. As described above, this test has great advantages with certain sera of relatively low titer not afforded by the intracerebral method.[63] Contrary to general belief, however, we have not found it more sensitive for detecting specific protection at a very low or equivocal level.[32] Although associated with disadvantages in regard to a suitable supply of infant mice, which most persons consider insuperable, actually it is an extremely useful modification which must be used occasionally in every laboratory.

4. *Technics*

a. Selection of virus strain Certain virus strains give higher neutralization indexes than others A strain should be selected which has this capacity, especially for the St. Louis viruses,[63] which all give relatively low neutralization indexes.

b. Preparation of virus. Approximately 10 inoculated mice are sacrificed after many or all show signs of infection The brains are removed aseptically and are ground in a sterile mortar with abrasive in cold, 50-per-cent-inactivated rabbit serum-saline or whole-rabbit serum inactivated at 56° C. for 30 minutes. This diluting fluid is added in such quantity as to make a 20 per cent suspension. The suspension is cen-

TABLE 4

TITRATION OF THE LD_{50} VIRUS DILUTION IN THE PRESENCE OF A PATIENT'S SERUM AND OF ITS CONTROL (FOR CALCULATION OF LD_{50} SEE TABLE 5)

Final dilution of virus	$10^{-1.4}$	$10^{-2.4}$	$10^{-3.4}$	$10^{-4.4}$	$10^{-5.4}$	$10^{-6.4}$*	$10^{-7.4}$	$10^{-8.4}$	$10^{-9.4}$
Dilution number	6	5	4	3	2	1	−1	−2	−3
Control rabbit serum	–	–	–	–	–	6/6†	4/6	1/6	0/6
Patient's serum	–	–	6/6	5/6	1/6	0/6	–	–	–

* [illegible] make the first dilution of the series, the decimal in the exponent of the dilution does not further complicate the calculation of the LD_{50} or the neutralization index

† The numerator indicates the number of mice dead the denominator, the number inoculated.

every test All tubes are incubated 2 hours at 37° C , chilled, and inoculated, using 4 mice for each unknown serum-virus mixture and 6 for each of the control dilutions If the control titration indicates that the dilutions were properly made, any serum protecting at least 3 or 4 mice (out of 4) in dilution 1 is considered tentatively as positive and scheduled for titration with the earlier serum or sera from the same patient. If too little virus has been used, as observed by the control titration, dilution 2 can possibly be considered significant If too much virus was used, only occasionally would a significant positive be missed if up to a 5- or 10-fold increase over the expected was employed. Nevertheless, under these circumstances, the test is repeated with those sera giving suspicious results (one or more mice protected).

e Comparative titrations of serum

(1) With serial dilutions of virus. If the titration is to be made using the more common method, serial dilutions of virus with undiluted frozen serum, the following technic is used. No change in procedure is necessary if all sera are inactivated at 56° C.[60] or if one chooses to dilute all sera with equal amounts of fresh or frozen antibody-free human serum to replace accessory substance which may have been lost.[32]

are inoculated per serum-virus mixture This is done under light ether anesthesia, 0.03 ml. in each, by the intracerebral route No antiseptic should be used on the mouse's head These mice are observed for an arbitrary number of days, depending on the incubation period and the pattern of deaths of the particular virus and mouse strain used. Ten days is usually an adequate period of time to observe practically all deaths that will occur from an intracerebral inoculation of a properly adapted virus in 3- to 4-week-old mice if a suitable, uniformly susceptible strain Deaths are recorded daily All dying in a period less than that of the minimal incubation period (determined by experience for each virus) are discounted as dying from extraneous causes. The LD_{50} (50 per cent mortality end point) is computed by the method of Reed and Muench[49] (see Table 5) The results of the tests on the three ampules are combined before calculating the LD_{50} or, after calculating each separately, the results are averaged. Following this preliminary titration with a "normal" serum to determine the LD_{50}, all unknown sera can be tested with a minimal number of dilutions

d. Screen test If series of sera are to be tested from several patients, it is usually advantageous, from the standpoint of economy, to test first only the final sérum from each patient to determine whether antibodies are present or absent If absent, it is unnecessary to test the earlier sera If antibody is apparently present, this will be confirmed by a titration including this and all earlier sera Thus, the results of a comparative titration are obtained

In general, for the screen test, two dilutions of virus are used, but the dilutions selected differ in various laboratories Since in some virus infections antibodies are slow to form, and, in some individuals do not occur in high titer at any time, it is now the practice in this laboratory to use two 10-fold dilutions, the higher dilution containing 10 to 25 LD_{50}. Dilutions are made in an identical manner to those used previously in the virus titration, except that the dilutions are started in such fashion that one (which will be called dilution 1) will represent between 10 and 25 LD_{50} In this laboratory, for practical purposes, we name the dilutions below 1 (mor [illegible] order 2, 3, 4, *etc.*, *and those above* 1 [illegible] −2, and −3 (see Table [illegible] are used with each unknown serum for the screen test. At the same time, dilutions 1, −1, −2, and −3 are added to an equal amount of normal rabbit serum for the control virus titration A control titration is made with

PATIENTS' SERUM

Dilution of Virus	Dilution No	Number Inoculated	Number Dead	Number Survived	Accumulated Tables: Died	Survived	Proportionate Mortality	Per Cent Mortality
$10^{-3\,4}$	4	6	6	0	12	0	12/12	100
$10^{-4\,4}$	3	6	5	1	6	1	6/7	86 (B)
$10^{-5\,4}$	2	6	1	5	1	6	1/7	14 (A)
$10^{-6\,4}$	1	6	0	6	0	12	0/12	0

(1) $\frac{50-14}{86-14}=\frac{36}{72}=$ 5 (C)

(2)
```
        5 4 (A)
      −   5 (C)
```
Patient's serum LD_{50} = 4 9

To compute neutralization index
LD_{50} in control serum 7 8
LD_{50} in patient's serum 4 9
Log of neutralization index 2 9
Neutralization index (antilog of 2 9) = 800

Calculation to Determine Number of LD_{50} in Dilution 1 ($10^{6\,4}$—*fold in this test*)
LD_{50} in control serum 7 8
Log in dilution 1 6 4
Log of difference 1 4
Number of LD_{50} in dilution 1 (antilog of 1 4) = 25

Since dilution 1 was calculated to fall between 10 and 25 LD_{50}, this titration was performed within a satisfactory range (25 LD_{50} used) Dilution 2 on the basis of this calculation was 250 LD_{50}

of the end-point dilution Thus, if the LD_{50} dilution in the presence of a normal control serum is $10^{-7\,8}$, the logarithmic expression of the LD_{50} is 7 8.*

The neutralization index of each serum (except those failing to protect against dilution 1) is obtained by subtracting its LD_{50} from that of the control and converting the difference to its antilog (Table 5). Thus,

* It is customarily assumed that this is a logarithmic expression and, simply stated [illegible] contained in the LD_{50} dilution

Serial dilutions are prepared as for the screen test or, in practice, the same set of dilutions is used, and some sera are set up for the screen test at the same time that the previously selected "positives" are titrated with their earlier paired serum or series of sera The number of dilutions to be used for each serum is one to be determined by judgment. Every serum is tested against dilutions 1, 2, and 3 at least, and late sera may be tested against one or more lower dilutions, particularly if the equine viruses are being used. Not less than 6 mice should be used for each dilution in the titrations if significant comparisons are to be made, although one may use 4 mice per dilution if 5-fold virus dilutions are employed. The LD_{50} virus dilution for each series of serum-virus mixtures and also that of the control are computed to 1 decimal point (Table 5). The logarithmic LD_{50} is expressed as the exponent of the reciprocal

TABLE 5

Calculation of the LD_{50} Dilution and the Neutralization Index of a Patient's Serum (from Titration in Table 4)

CONTROL SERUM								
					Accumulated Tables			
Dilution of Virus	Dilution No	Number Inoculated	Number Dead	Number Survived	Died	Survived	Proportionate Mortality	Per Cent Mortality
$10^{-6\,4}$	1	6	6 ↑	0 ↓	11	0	11/11	100
$10^{-7\,4}$	−1	6	4	2	5	2	5/7	71 (B)
$10^{-8\,4}$	−2	6	1	5	1	7	1/8	12 (A)
$10^{-9\,4}$	−3	6	0	6	0	13	0/13	0

Compute as follows·

(1) $\dfrac{50\% - \%\text{ mortality of first dilution with} < 50\%\text{ mortality (A)}}{\%\text{ mortality of first dilution with} > 50\%\text{ mortality (B)} - \%\text{ mortality in (A)}}$

= Factor of proportionate distance (C)

$$\frac{50-12}{71-12} = 6\ (C)$$

(2) Log of reciprocal of first dilution with <50% mortality (A) 8 4
(subtract) Proportionate distance factor (C)×log of dilution factor (10)
which is 1— .6
Control Serum LD_{50} = 7 8

75 LD_{50}) is employed except for the control titration with normal undiluted rabbit serum. This control series must extend from dilution 1 through –3.

The LD_{50} in each serum is computed again by the method of Reed and Muench, now on the basis of serum dilutions It must be recalled when making the calculations that the logarithm of the dilution factor for a 5-fold dilution is 7, not 1, which was used in 10-fold dilutions (see computation (2) in upper part of Table 5) This measure has no absolute value as it had in the previously described test for it depends entirely on the amount of virus present in the test dose However, two sera from the same patient tested simultaneously are readily compared Comparison is expressed as the arithmetic quotient of the reciprocals of two LD_{50}. For example, if the LD_{50} in the first serum is 1 25 and that in the second is 1 625, there is a 25-fold increase If that of the first is 1·10 and that of the convalescent specimen 1·32, the apparent change is 3 2-fold

(3) Intraperitoneal neutralization test For performing this test it is recommended that the technic of Olitsky and Harford[46] or that of Lennette and Koprowski[47] be employed However, incubation of the serum-virus mixture for 2 hours at 37° C prior to inoculation will increase the sensitivity of the test[32] This type of test is preferred by certain workers[63] for the diagnosis of St Louis virus infections since antibody rises are frequently difficult to demonstrate by the intracerebral test Either serial dilutions of serum or virus may be used, and calculations are made in the same manner as described for the intracerebral tests

5 *Interpretation* Because of antigenic differences in strains of the same virus, of marked differences in degree of response and time of development of antibodies with different viruses, and of the lability of antibody under certain storage conditions, it is impossible to set lower limits for a significant positive response In a St Louis infection, a neutralization index of 20 (by intracerebral test) in a 20-day serum, using one strain of virus, might be highly significant if the test result is adequately confirmed by repeat tests On the other hand, a neutralization index of 20 in another patient (Western equine infection), with at least one strain of Western equine virus, in a serum taken 20 days after onset would usually be considered as of no significance The interpretation of a change between an acute phase and a convalescent serum cannot be made from a table or b[illegible]
too many [illegible]
with the s[illegible]
technical [illegible]
matters of temperature and time of storage of sera, and an understanding of the

if the computed LD_{50} dilution in the control is $10^{-7.8}$ (LD_{50} is 7.8) and that in the unknown serum $10^{-4.9}$ (LD_{50} is 4.9), their respective difference is 2.9 and the antilog or neutralization index 800. If the control LD_{50} is 7.9 and that of the unknown 7.4, the difference by subtraction is 0.5, the antilog of which (neutralization index) is 3.2. Table 4 gives an example of the titration of a serum and its control, Table 5 the calculation of the LD_{50} dilution and the neutralization index. Calculation of the number of LD_{50} doses actually occurring in dilution 1 in this experiment is also included. This amount is essential to know, in case sera were included for the screen test. It will be observed that the test results (dilution 1) were within the required limits (10-25 LD_{50}). In experienced hands, the range of variation is usually well under 10-fold (one log).

Differences of less than 10-fold in the neutralization index between two sera are usually not considered to be significant although differences of 5-fold are frequently repeatable in experienced hands and may have significance.

(2) With serial dilution of sera. If this method is selected there is no change in the manner of handling the frozen virus, and the same method of preliminary titration and titration of the control serum is employed.

Sera are set up in 5-fold dilutions, and a constant amount of virus is added to each serum dilution. All serum-virus mixtures are incubated at 37° C for 1 hour before inoculation. Two hours of incubation is not recommended when high dilutions of serum are employed. The calculations of the LD_{50} are made on the basis of serum dilutions.

It is usually advisable to employ about 4 serum dilutions with an acute phase serum, and 6 with a convalescent. The number of days after onset when the serum was collected and the usual time for antibodies to form with the virus in question should be considered as factors influencing choice of dilutions. For dilutions of each serum, wide-mouth short tubes are selected. In each of the first two of a series is placed 0.2 ml of the serum to be tested. To each but the first is added 0.8 ml of a 10 per cent serum-saline diluting fluid. After mixing the contents of the second tube, 0.2 ml is transferred to the third, etc., in series, and 0.2 is finally discarded from the last. Virus is added to these same tubes; 0.2 to the tube of undiluted serum and 0.8 to all others. In this manner, a minimum of serum and glassware is required. Virus dilutions are prepared as in the other test. Only one dilution (preferably between 125 and

antigens may be prepared from infected guinea pig spleen[73] or from the chorioallantoic membrane of infected chick embryos[73a] With some of the encephalitic viruses, especially Western equine[70,71] and Murray Valley,[2] membranes or fluids from infected chick embryos have been found satisfactory. The embryo itself is not a satisfactory source of antigen since it occasionally reacts with normal human sera Antigens prepared with membranes or fluids have the advantage of being easier and safer to prepare No satisfactory antigen has been prepared for St. Louis or Japanese B viruses from egg membranes or fluids of chick embryos Therefore, since controls of each type of tissue used for antigen preparation must be included in each test, the advantages of using a single type

TABLE 6
AVERAGE HUMAN IMMUNOLOGIC PATTERN IN CERTAIN VIRUS INFECTIONS

Virus	Type Test	Weeks after Onset 0-1	1-2	2-3	3-4	7-8	15-16	1 yr	2 yrs
Western equine	Neut	++	++++	+++++	+++++	+++++	+++++	++	+
	C F	0	±	+	++	+++	++	±	0
St. Louis	Neut	0	±	+	++	++	+++	++	+
	C F	±	+	+++	++++	++++	+++	+	±
Japanese B	Neut	±	++	+++	+++	++++	++++	++	+
	C F	±	++	+++	++++	+++	+++	+	?
Lymphocytic choriomeningitis	Neut	0	0	±	±	+	++	+	+
	C F	0	+	++	++	++	++	±	0

of tissue are easily recognized, provided there is not a loss in sensitivity in the test

In general, the complement fixation test made for the purpose of diagnosis should include several different antigens, since it is not possible to differentiate the etiologic type of encephalitis on a clinical basis. It is both customary and advisable to test a serum against 3 to 6 antigens This will not only improve the likelihood of determining the specific causative agent, but the additional antigens will serve as controls Unfortunately, at this time, the geographic distribution of many of these agents is still unknown, so that one cannot rely on a knowledge of the agents recognized to be active in an area On specimens originating in the United States, it is practical to include antigens to Western equine, Eastern equine, St Louis, lymphocytic choriomeningitis, mumps, and poliomyelitis.

Sera for this test must be collected using the same precautions as

usual and extremes of human immunologic response to the virus infection in question Several of these infections have distinctly individual immunologic patterns so that no generalization is permissible Table 6 will give some helpful comparative data on four of these viruses The complement fixation response is included in the same table Personal experience provides reasonable assurance of the general accuracy of the pattern only with the Western equine, Japanese B, and St Louis viruses The pattern of lymphocytic choriomeningitis antibodies is based largely on the literature The Eastern equine virus probably behaves much like the Western so far as the neutralization test is concerned

One fact must be emphasized in interpretation In infections with the four viruses listed in Table 6 at least, no significance can be placed on any positive neutralization result with a single convalescent serum Mild missed cases are known to occur frequently, and in certain communities a considerable proportion of normal residents have antibodies to one or several of these viruses [52] A definite rise in titer between two suitably spaced sera must be obtained, and the significance of the rise must be evaluated on the basis of several facts, including the time of onset, day of collection of sera, and temperature of storage of the serum at all times These data must be available to the laboratory director if he is to interpret the results of the tests If the physician attempts to interpret the tests, he must have as thorough an understanding of the laboratory procedures and behavior of the animals and virus strains as the laboratory director.

A negative neutralization test result probably has more clear-cut diagnostic significance than any other test result, at least when as little as 10 or 25 LD_{50} of virus is used (see Table 6) and the serum is taken 2 months or more after the onset of illness Individual cases are on record where neutralizing antibodies have apparently failed to develop, but in most instances a negative result by this one test has unchallenged significance no infection

Of inestimable value in interpretation, in many instances, are the additional results of the complement fixation and hemagglutination inhibition tests performed on the same sera If there is any doubt, several tests should be performed

D. Complement Fixation Test

1. *General discussion* With the recent advances made in preparation of antigen and in test technics, and with accumulated experience by extensive use in several laboratories, the complement fixation test has become a very useful diagnostic aid for this group of diseases [70] A more clear-cut diagnosis can be made in the majority of cases of disease caused by several common members of this group than by the neutralization test. For a few, an earlier diagnosis frequently can be made (Table 6).

The chief problem in complement fixation is to obtain a satisfactory antigen. It is now possible to prepare relatively stable, sensitive antigens from infected mouse brains by extraction of the material with lipid solvents to remove substances which formerly caused undesirable nonspecific reactions [71,72] For lymphocytic choriomeningitis, satisfactory

the time required to prepare the antigen and of requiring only ordinary laboratory equipment for its preparation These antigens appear to be nonanticomplementary, specific, and reliable Several workers have found this a satisfactory method for the preparation of antigen[23] It must be emphasized that by either this method or the benzene method, highly infectious, active virus particles may be present immediately after preparation since no procedure for virus inactivation has been employed It may be possible to inactivate with ultraviolet light,[75] but this is not done routinely for either of these methods Use of live antigens constitutes a real hazard, however Since our greatest experience has been with benzene extraction, the currently used modification of the method described by España and Hammon is given[71]

Mice are inoculated intracerebrally with a 10^{-2} or 10^{-3} dilution of virus The mice, when most are moribund, are anesthetized, bled to death, and the brains removed The brains are then ground in a Waring blendor in pyrogen-free, fractionally distilled water to make a 20 per cent suspension This is done under a hood, protected with ultraviolet light Twenty-five to 50 ml amounts are transferred to 250 ml Pyrex bottles, when it is considered safe to open the blendor, rapidly shell-frozen, and lyophilized overnight in a specially constructed apparatus with a Megavac pump and no exhaust apertures less than 5⁄8-inch in diameter The next stages are dangerous and should be carried out under a hood in a closed system The dried tissue is then extracted with benzene for 1 hour at room temperature by adding to the 250 ml bottle a volume of benzene equivalent to twice the original aqueous suspension This is stoppered and shaken several times during the hour The material is poured into a Gooch crucible filter (sintered glass of porosity M) fitted to a filter flask and the benzene rapidly removed by vacuum The filter is covered with a petri dish Two further benzene extractions are performed in the same filter, allowing 30 minutes at room temperature each time before applying vacuum The material is stirred while covered with benzene and more benzene added if necessary After the last extraction, while still under vacuum, the cake is carefully broken up with a spatula The remaining solvent is removed by placing the entire filter (petri dish over top of filter) in a desiccator and by applying vacuum for about 30 minutes The dry powder is next transferred to a mortar (This operation is very dangerous, since the dry powder is highly infectious) A few ml of saline are added to the dry powder, the powder allowed to absorb the saline, and a smooth paste prepared Finally it is resuspended to the original volume in buffered saline, pH 7.38, transferred to a flask, stoppered, and rehydration permitted to continue overnight at 5° C It is next centrifuged for 1 hour at 10,000 r p m at 0° C The supernatant is removed, and merthiolate is added to a final dilution of 1 10,000 It is stored in rubber-stoppered vaccine bottles at 5° C This antigen is ready for immediate use Antigens prepared and stored in this way have remained satisfactory for at least 1 year When it is desired to ship these antigens, the dry powder obtained after benzene extraction may be suspended in distilled water, centrifuged, and the supernatant lyophilized The lyophilized material, powder form, goes readily into solution

for the neutralization test. It is advisable to have two or more serum specimens, collected at intervals of 10 or more days, stored under ideal conditions and tested simultaneously

Since the time of appearance and wane of complement-fixing antibodies is frequently entirely different from that of neutralizing antibodies (see Table 6), these two tests may show some interesting differences in results which, when understood, are of great practical value. In general, complement-fixing antibodies in this group of infections disappear or drop to a very low level by 1 to 2 years after the infection while neutralizing antibodies remain elevated Therefore, the presence of one type of antibody in the absence of the other may have considerable significance in epidemiologic or diagnostic studies. If, for example, only one serum, taken on the 12th to 20th day, was available in a case of St Louis infection, the confirmed presence of complement-fixing antibody without neutralizing antibody would probably mean a very recent infection with St. Louis virus or one antigenically related to it. Conversely, the presence of neutralizing antibody in the absence of complement-fixing antibody in this serum would indicate experience with this virus one or more years before, but not currently.

For many of these viruses, it is now possible to obtain antigens and control hyperimmune serum from commercial companies. If these reagents prove satisfactory the problem of the diagnostic laboratory is greatly simplified For those who desire or who must make their own antigens the following technic is recommended

2. *Preparation of antigens.* Antigens for most of these viruses are prepared by extraction with lipid solvents, using either lyophilized material[71] or an aqueous suspension[72] This lipid extraction has eliminated much of the nonspecific and anticomplementary effects noted with antigens prepared by other methods. Benzene extraction of antigens was introduced by DeBoer and Cox.[74] Extraction removed much of the fraction which reacted with Wassermann positive sera and also lowered the infectious titer. More recently certain modifications of their methods have been developed by España and Hammon[71] These simplify the procedure, shorten by several days the time required for preparation of the benzene-extracted antigen, and result in an antigen with very high titer and greater sensitivity. When used in optimal dilutions they are both more sensitive and more specific than the unextracted antigens. Still more recently, Casals developed a method of acetone-ether extraction[72] This method has the advantage of shortening to less than a day

For rapid tentative identification of freshly isolated strains of the arthropod-borne encephalitis viruses, it has been found helpful to prepare a complement-fixing antigen with the new isolate and test it against known hyperimmune sera For this purpose, it is not necessary to extract the tissue with lipid solvents Instead, the following method, a modification of that by Havens and his coworkers,[77] has been found satisfactory. Mice, at least 3 to 5, moribund after inoculation with the unknown virus, are bled out The brains are removed and ground in a Waring blendor or mortar A 10 per cent brain suspension is prepared in a 2 per cent guinea pig serum (inactivated at 56° C for 30 minutes)-saline. The suspension is centrifuged for 1 hour at 16,000 r p m at 0° C. The supernatant is the antigen and is used undiluted It may be used on the same day prepared A control antigen is prepared at the same time by the same method from normal mouse brains and is included in the test In many cases, a new virus, tested in this way, may be tentatively identified sooner than would be possible by mouse neutralization tests. Some newly isolated viruses do not make satisfactory antigens, however, and other methods of identification must be used (See section II, J)

3 *Preparation of control hyperimmune sera* Casals[79] recommends the following method of preparing mouse sera

(1) Animals are immunized with homologous tissue in order to prevent the formation of organ- and species specific antibodies (2) the nonspecific tissue reaction is eliminated by a) centrifuging antigens at sufficiently high speed so as to sediment all or most of the tissue component (Havens *et al*[77]) or b) inactivating the sera at 60 to 65° C, according to species, thus destroying the thermolabile serum substance while hardly affecting the specific virus antibody[65]

The immunization of animals can be carried out by means of 2 intraabdominal injections of a 10 per cent formolized brain suspension, followed within 10 days by an intraabdominal injection of active virus in dilution of 10^{-2}, repeated every 4 or 5 days Within 4 to 6 weeks most animals develop a high-titer serum, which can be maintained by subsequent injections

Guinea pig or hamster sera are used in this laboratory [79] Formalinized homologous brain tissue is used as inoculum for the equine viruses For the others, two intracerebral inoculations of hamster brain virus result in high titer sera. These sera must be kept frozen, otherwise the titer falls rather rapidly

4 *Technic of test* Two technics will be described for the complement fixation test. The first is the España and Hammon modification of that described by Casals[78] The quantity of each reagent used

and requires no centrifugation prior to use. The titer is the same as before lyophilization

Antigens prepared thus usually have a titer of 32 to 64 units With *hyperimmune* sera, highest serum titers are obtained using the antigen in a 1:16 to 1 32 dilution (2 or 4 units), but for *human* sera of lower titer the optimal dilution of antigen appears to be from 1:4 to 1:8 (8 or 16 units). Greater excess of antigen tends to inhibit the reaction. As demonstrated by DeBoer and Cox,[74] these extracted antigens do not give a nonspecific reaction with Wassermann positive sera which have been inactivated at only 60° C.

Antigens are not inactivated in this laboratory although the freshly prepared antigens may be quite infectious, titering only 3 to 4 logs less than the original virus suspension. This infectivity is gradually lost, however, after continued storage at 5° C.

For preparation of lymphocytic choriomeningitis antigens from guinea pig spleens, the original procedure described by Smadel *et al*,[73] is satisfactory: "The antigen is most readily obtainable from spleen (of acutely ill guinea pigs) and least from brain, splenic suspension containing 1 part (dry weight) in 6,000 gives complete fixation. Ten minutes in a concentration centrifuge (Bauer and Pickels) at 20,000 r p m and the supernatant fluid is filtered through a Seitz pad. Such preparations of spleen are not anticomplementary after storage, the contained antigen is little affected by storage at 3° C for 6 months, by heating at 56° C for ½ hour, by variations in pH range from 4.5 to 9, or by dialysis." Antigens freshly prepared from guinea pig spleens react nonspecifically with a number of human sera This nonspecific factor is lost after storage for several weeks. For the past 5 years Smadel (personal communication[76]) has concentrated and partially purified the splenic antigen in the following manner. "After high-speed centrifugation and Seitz filtration the solution is brought to pH 4.8–5.0 with N/10 HCl and the resultant flocculant precipitate removed by horizontal centrifugation and discarded The solution is promptly readjusted to pH 7 0 and placed in a viscose tube in front of a fan at room temperature until concentrated by perevaporation to one-fourth the original volume. Such preparations generally have titers of 1/128. Sufficient antigen to last for several years is usually prepared at one time and stored in 5–10 cc. amounts at –20° C. or in the lyophilized state Portions of this stock which are employed for current testing may be stored at 5° C. for several months."

according to the method of Mayer, Osler, Bier, and Heidelberger[84] as follows: Stock Dissolve 5 75 gms of 5 5-diethyl barbituric acid (barbital) in 500 ml hot demineralized (or glass) distilled water Add 3 75 gms Na-5 5-diethyl barbituric acid (Na barbital) and 85 gms NaCl Make up to 2,000 ml with demineralized (or glass) distilled water Add 1 68 gms $MgCl_2 6H_2O$ and 0 28 gms $CaCl_2$ Autoclave at 15 lb /sq in for 20 minutes Final pH 7.2 Store at 4° C

For use, dilute the stock 1/5 with demineralized (or glass) distilled water

(5) Sera The sera to be tested are inactivated for 20 minutes in a 1 4 dilution at 60° C for human or mouse, 62° C for hamster, or 56° C for guinea pig If anticomplementary, it is sometimes possible to eliminate this by inactivation at 65° C for 20 minutes or by inactivating on 2 successive days at 60° C If this treatment is inadequate the serum is unsuitable for use The tests are set up as serum titrations Series of 2-fold serum dilutions are prepared for each antigen used in the test.

(6) The test All reagents are held in an ice-water bath while the test is being set up The reagents are mixed in the following order 0 2 ml serum dilution, 0 2 ml (2 units) complement, and 0 2 ml antigen at the "optimal" dilution as determined previously by a "box titration" in the presence of homologous hyperimmune serum These mixtures are incubated in the refrigerator (2–4° C) for 16 to 18 hours[85] and then put in a water bath at 37° C for one-half hour Next, the sensitized cells (prepared the previous day and held in the refrigerator until the test was removed and then held at room temperature) are added in 0 4 ml amounts to each tube The tubes are incubated then for one-half hour at 37° C and read Appropriate controls for each reagent are included with each test

b Plate method

(1) Equipment

(a) Plates Plastic plates ruled in ¾-inch squares 14 across and 12 down were made to fit into large enamel refrigerator pans Plates are cleaned by immersion in a weak (0 2%) hydrochloric acid solution for several hours The surface is then cleaned with a mild detergent (for example, Dreft), using a sponge to rub the surface, and then rinsed well first under running, cold tap water, followed by running distilled water They are air-dried at room temperature It is important not to rub the dry sheets with a cloth because they easily become electrically charged, thus dragging the drops off the end of the pipette by the electrostatic attraction before they have grown to the size expected under the pull of gravity alone[89]

(b) Racks for plates In this laboratory air-tight containers were made from refrigerator pans by covering the pans with parafilm and sealing any openings with adhesive tape One pan is kept in the refrigerator and one in the 37° C incubator A layer of moistened absorbent cotton is placed in the bottom of each pan Large rubber stoppers (at least ⅝ inch long) placed at each corner are used to support the plates Three plates stacked each above the other and separated by rubber stoppers will fit into one pan

(c) Dropping pipettes The dropping pipettes are made, using 19-gauge needles cut off straight and attached to glass "observation" tubes (used ordinarily to connect an intravenous needle to rubber tubing) A 1 0 ml rubber bulb fitted to the other end of the "observation" tube is used to aspirate the reagent and to deliver the

is 0 2 ml, and the final volume in each tube is 1 0 ml. The second is a modification of the drop or plate method of Fulton and Dumbell[80] This latter method has been adapted by Svedmyr, Enders, and Holloway to the poliomyelitis viruses[81] and by Kraft and Melnick to the Coxsackie viruses,[82] and has been found satisfactory in this laboratory for the encephalitis viruses[32] It has the *distinct* advantage that the quantities of reagent required are minimal since the final volume is 0 10 ml, thus resulting in a great saving of both serum and antigen

a Test tube method

(1) Hemolysin The unit of hemolysin used in this test is 0 2 ml of the highest dilution of antisheep rabbit serum giving complete hemolysis of 0 2 ml of a 2 per cent suspension of washed, packed sheep cells in the presence of 0 2 ml of 1 30 dilution of complement after incubation at 37° C for 30 minutes

(2) Hemolytic system Sheep blood is collected aseptically and either treated with citrate or with an equal quantity of Alsever's solution[83] and stored in the icebox The amount required for the test is removed with a sterile pipette, and the red cells washed three times with saline After the last washing, cells are measured, and a 2 per cent suspension made Equal volumes of this cell suspension and a dilution of hemolysin containing 2 units in 0 2 ml constitutes the hemolytic system This is mixed thoroughly and held at room temperature for 15 minutes before use in the test A portion of this same sensitized cell suspension, held overnight at 5° C., may be used to complete the test on the following day

(3) Complement Either lyophilized complement, obtained from a commercial source (if the titer is at least 1/30) or frozen, ampuled guinea pig sera, representing a pool of from 10–15 guinea pigs may be used A preliminary titration of the complement is made in saline and also in the presence of each antigen Table 7 will serve

TABLE 7
COMPLEMENT TITRATION TUBE METHOD

Tube No	1/30 Comp ml	Saline ml	Antigen or Saline ml	Incubation	Sensitized Cells ml	Final Incubation
1	0 05	0.35	0 20	37° C 1 hr	0 40	37° C 30 min
2	0 08	0 32	0 20		0 40	
3	0 10	0 30	0 20		0 40	
4	0 12	0 28	0 20		0 40	
5	0 14	0 26	0 20		0 40	

as a guide for the complement titration The complement unit is the highest dilution giving complete hemolysis Two units in 0 2 ml are used in the test proper At the same time this preliminary titration is made, a duplicate titration of complement with antigen is set up and incubated in the refrigerator along with the test proper.

(4) Saline Veronal buffer, pH 7 2, is used as the diluent This is prepared

placed immediately in the pan which has been previously chilled, covered with parafilm, and placed in the refrigerator They are held for 16 to 18 hours, then removed from the refrigerator and from the pan Water of condensation, if present, is allowed to evaporate (15 minutes at room temperature) and 2 drops of sensitized cells (40 mm ^{3}) added The plates are then incubated 1 hour at 37° C in a second air-tight container, then removed from the incubator, water of condensation (if any) allowed to evaporate, and read

The test is read by placing the sheet on a white background with a strong overhead light Where the cells have not been hemolyzed they collect in a central dot The end point is taken as that square which by inspection shows approximately half the cells hemolyzed (2 +) If the cells are not confined to the central dot, it has been found that gentle tapping of the plate or twisting the plate in a slight rotary motion will clarify the pattern Also, as the plate stands, the pattern will become more pronounced Placing plates in the refrigerator for 15 to 20 minutes also aids in pattern formation

5. *Interpretation.* For diagnostic purposes it is necessary to test simultaneously two or more serial bleedings on a person In the majority of cases, a significant rise in titer will be demonstrated In general, a 4-fold rise is considered significant When interpreting the results it is necessary to consider both the time of collection with respect to the illness and the interval between collections. The time and degree of rise will usually be in rough accordance with that shown in Table 6 If possible, it is advisable to perform both the complement fixation and the neutralization test since, in some instances, a rise in titer may be demonstrated with one test and not with the other

IV REFERENCES

1 Hammon, W McD, and Reeves, W C Recent Advances in the Epidemiology of the Arthropod Borne Virus Encephalitis Including Certain Exotic Types *Am J Pub Health*, 35 994–1004, 1945

2 French, E L Murray Valley Encephalitis Isolation and Characterization of the Aetiological Agent *M J Australia*, 1 100–103, 1952

3 Hammon, W McD, and Reeves, W C California Encephalitis Virus, A Newly Described Agent I Evidence of Natural Infection in Man and Other Animals *California Med*, 77 303–309, 1952

4 Hammon, W McD, Reeves, W C, and Sather, G California Encephalitis Virus, A Newly Described Agent II Isolations and Attempts to Identify and Characterize *J Immunol*, 69 493–510, 1952

5 Reeves, W C, and Hammon, W McD California Encephalitis Virus, A Newly Described Agent III Mosquito Infection and Transmission *J Immunol.*, 69 511–514, 1952

6 Casals, J, and Webster, L T Relationship of the Virus of Louping Ill in Sheep and the Virus of Russian Spring-Summer Encephalitis in Man *J Exper Med*, 79 45–63, 1944

7 Pond, W L, Russ, S B, and Warren, J The Russian Spring-Summer Encephalitis and Louping Ill Group of Viruses, Relationship of European and Asiatic Strains of Russian Spring Summer Encephalitis Viruses and Louping Ill Virus *J Infect Dis.*, 93 294–300, 1953.

drops The pipettes are each carefully tested, and only those needles delivering 20 mm^3 (0 02 ml or 50 drops/ml) are used.

(2) Saline. Veronal buffer as described for tube test

(3) Sensitized sheep cells. Sheep cells are prepared in essentially the same manner as for the tube test except that the final concentration is 0 4 per cent

The hemolysin titration is performed in test tubes, using 0.2 ml. quantities of each reagent, in the same manner as for the tube test except that 0 4 per cent sheep cells are used The highest dilution showing crystal-clear hemolysis is used as the end point and represents 1 unit of hemolysin

The sensitized cell suspension is prepared on the 1st day of the test by adding an equal quantity of a dilution of hemolysin representing 4 units to the 0 4 per cent cell suspension and handled as previously described Sensitization is allowed to proceed for 30 minutes at 37° C and the flask is then placed in the refrigerator, ready for use the next day Just prior to use it is warmed to room temperature

(4) Complement A preliminary complement titration should be done once with each new lot of complement to determine the approximate range of reactivity and the dilution representing 2 to 3 units In this laboratory it is convenient to use the standard test tube method, 1 hour incubation at 37° C and 0.2 ml. amounts (see tube method). In our experience, most lots of complement can be used in a dilution of 1/60 For the test itself, the complement titer in the presence of each antigen and saline is verified and the number of units determined by inclusion of a titration with each test run A dilution of complement, representing 2 to 3 units as determined by previous titration, is used in the test proper The dilutions of complement are prepared according to the protocol in Table 8.

TABLE 8

COMPLEMENT TITRATION PLATE METHOD

Tube	Reciprocal of Complement Dilution	Complement ml.	Diluent ml
1	30	0 3 (und)	8.7
2	60	2 0 (1)	2 0
3	75	2 0 (2)	0.5
4	94	2 0 (3)	0 5
5	117	2 0 (4)	0.5
6	146	2 0 (5)	0 5
7	182	2 0 (6)	0 5
8	227	2 0 (7)	0 5
9	284	2 0 (8)	0.5
10	355	2 0 (9)	0 5

(5) Sera Serial 2-fold dilutions of inactivated sera are prepared *in test tubes* as in the tube test

(6) Conduct of test The reagents are added to the plate as follows 1 drop (20 mm^3) serum dilution, 1 drop complement dilution representing 2 to 3 units, 1 drop antigen (optimal dilution) For the complement titration, 1 drop of saline is substituted for the serum No mixing is necessary The pipette is held in a vertical (90°) position while dropping reagents. Each plate, after all reagents are added, is

26 Hammon, W McD The Encephalitides of Virus Origin with Special Reference to Those of North America *Clinics*, 4 485-503, 1945

27 Haymaker, W, and Smadel, J E The Pathology of the Viral Encephalitides *Publ Army Medical Museum*, 1943

28 Zimmerman, H M Pathology of Japanese B Encephalitis *Am J Path*, 22 965-991, 1946

29. Lillie, R D The Pathology of Lymphocytic Choriomeningitis Virus Infection A Discussion, Chapter 13 in *The Pathogenesis and Pathology of Viral Diseases*, edited by John G Kidd New York, Columbia Press, 1950, pp 181-193

29a Wolf, A The Pathology of Some Viral Encephalitides Chapter 14 in *The Pathogenesis and Pathology of Viral Diseases*, edited by John G Kidd New York, Columbia Press, 1950, pp 194-213

30 Sanders, M, Blumberg, A, and Haymaker, W Polyradiculopathy in Man Produced by St Louis Encephalitis Virus *South M J*, 46 606-608, 1953

30a Haymaker, W, Sather, G E, and Hammon, W McD Accidental Russian Spring-Summer Viral Encephalitis *Arch Neurol and Psychiat*, 73 609-630, 1955

31 Reeves, W C The Encephalitis Problem in the United States *Am J Pub Health*, 41 678-686, 1951

31a Smithburn, K C, and Bugher, J C Ultrafiltration of Recently Isolated Neurotropic Viruses *J Bact*, 66 173-177, 1953

32 Hammon, W McD, and co-workers Unpublished data

33 Hammon, W McD, and Reeves, W C Certain Bacteriostatic Agents Added to Sera Used in Diagnostic Tests for Neurotropic Virus Infections *Proc Soc Exper Biol and Med*, 60 84-88, 1945

33a Rogers, N G The Effect of Merthiolate on the Infectivity of Certain Viruses *J Lab and Clin Med*, 38 483-485, 1951

34 Hammon W McD, Reeves, W C and Izumi, E M Mosquitoes and Encephalitis in the Yakima Valley, Washington II Methods for Collecting Arthropods and for Isolating Western Equine and St Louis Viruses *J Infect Dis*, 70 267-272, 1942

35 Bang, F B, and Herriott, R M Purification of Equine Encephalomyelitis Virus by Ultracentrifugation and Maintenance of Its Activity with Cysteine *Proc Soc Exper Biol and Med*, 52 177-180, 1943

36 Labzoffsky, N A Effect of Reducing Agents on the Viability of Equine Encephalomyelitis Virus (Eastern Type) *Canad J Res, Sect E, Med Sci*, 24 119-133, 1946

37 Taylor, A R, Sharp, D G, and Beard, J W Influence of pH on the Molecular Stability of the Equine Encephalomyelitis Virus Protein (Eastern Strain) *J Infect Dis*, 67 59-66, 1940

38 Duffy, C E, and Stanley, W M Studies on the Biochemical, Biophysical, and Immunogenic Properties of Japanese B Type Encephalitis Virus and Vaccines *J Exper Med*, 82 385-410, 1945

39 Duffy, C E pH Stability of St Louis Encephalitis Virus *Proc Soc Exper Biol and Med*, 63 333, 1946

40 Olitsky, P K, Casals, J, Walker D L, Ginsberg, H S, Horsfall, F L, Jr Preservation of Viruses in a Mechanical Refrigerator at -25° C *J Lab and Clin Med*, 34 1023-1026, 1949

41 Sulkin, S E, and Pike, R M Viral Infections Contracted in the Laboratory *N Eng J Med*, 241 205-213, 1949

42 Beard D W, Finkelstein H and Beard J W Repeated Vaccination of Man Against the Virus of Equine Encephalomyelitis *J Immunol*, 40 497-507, 1941

42a Smadel, J E The Hazard of Acquiring Virus and Rickettsial Diseases in the Laboratory *Am J Pub Health*, 41 788-795, 1951

43 Webster, L T Inherited and Acquired Factors in Resistance to Infection II A Comparison of Mice Inherently Resistant or Susceptible to Bacillus Enteritidis Infection with Respect to Fertility, Weight, and Susceptibility to Various Routes and Types of Infection *J Exper Med*, 57 819-843, 1933

8. Smithburn, K C Differentiation of the West Nile Virus from the Viruses of St Louis and Japanese B Encephalitis *J Immunol*, 44 25–31, 1942
9. Melnick, J L, Paul, J R, Riordan, J T, Barnett, V. H, Goldblum, N, and Zabin, E. Isolation from Human Sera in Egypt of a Virus Apparently Identical to West Nile Virus *Proc Soc Exper Biol and Med*, 77 661–665, 1951.
10 Bernkopf, H, Levine, S, and Nerson, R Isolation of West Nile Virus in Israel *J Infect Dis*, 93 207–218, 1953
11 Goldblum, N, Sterk, V V., and Paderski, B West Nile Fever The Clinical Features of the Disease and the Isolation of West Nile Virus from the Blood of Nine Human Cases *Am J Hyg*, 59 89–103, 1954
12. Work, T H, Hurlbut, H S, and Taylor, R M Isolation of West Nile Virus from Hooded Crow and Rock Pigeon in the Nile Delta *Proc Soc Exper. Biol. and Med*, 84 719–722, 1953
13 Taylor, R. M, and Hurlbut, H S, with the technical assistance of Dressler, H R, Spangler, E W, and Thrasher, Del Isolation of West Nile Virus from Culex Mosquitoes *J Roy Egyptian M A*, 36 199–208, 1953.
14 Dick, G W A, Best, A M, Haddow, A J, and Smithburn, K. C Mengo Encephalomyelitis, Hitherto Unknown Virus Affecting Man *Lancet*, 2 286–289, 1948
15 Warren, J, Smadel, J E, and Russ, S B The Family Relationship of Encephalomyocarditis, Columbia-SK, M M, and Mengo Encephalomyelitis Viruses *J Immunol*, 62 387–398, 1949
16. Smithburn, K C, Mahaffy, A F, and Paul, J H Bwamba Fever and Its Causative Virus *Am J Trop Med*, 21 75–90, 1941
17. Smithburn, K C, and Haddow, A J Semliki Forest Virus I Isolation and Pathogenic Properties *J Immunol*, 49 141–157, 1944
18 Roca-Garcia, M The Isolation of Three Neurotropic Viruses from Forest Mosquitoes in Eastern Columbia *J Infect Dis*, 75 160–169, 1944.
19. Smithburn, K C, and Haddow, A J Ntaya Virus. A Hitherto Unknown Agent Isolated from Mosquitoes Collected in Uganda *Proc Soc Exper. Biol and Med.*, 77 130–133, 1951
20. Dick, G W A, and Haddow, A J Uganda S Virus A Hitherto Unrecorded Virus Isolated from Mosquitoes in Uganda I Isolation and Pathogenicity. *Tr Roy Soc Trop Med and Hyg*, 46 600–618, 1952
21 Dick, G W A, Kitchen, S F, and Haddow, A J Zika Virus I Isolations and Serological Specificity *Tr Roy Soc Trop Med and Hyg*, 46 509–520, 1952.
21a Macnamara, F N Zika Virus A Report on Three Cases of Human Infection During an Epidemic of Jaundice in Nigeria *Tr Roy Soc Trop Med and Hyg*, 48.139–145, 1954
22. [illegible] in the Tropics of Africa and [illegible] mined by Cross-Neutralization
23 F [illegible] Tropics of Africa and South America Immunological Reactions as Determined by Cross-Complement Fixation Tests *J Immunol*, 68.461–472, 1952
24 Taylor, R M Studies on Certain Viruses Isolated in the Tropics of Africa and South America Their Growth and Behavior in the Embryonated Hen Egg *J Immunol*, 68 473–494, 1952.
25 Dick, G W. A Epidemiological Notes on Some Viruses Isolated in Uganda (Yellow Fever, Rift Valley Fever, Bwamba Fever, West Nile, Mengo, Simliki Forest, Bunyamwera, Ntaya, Uganda S and Zika Viruses) *Tr Roy Soc. Trop Med and Hyg* 47 13–48, 1953
25a. Laemmert, H. W, Jr, and Hughes, T P The Virus of Ilhéus Encephalitis Isolation, Serological Specificity and Transmission *J Immunol*, 55 61–68, 1947
25b Taylor, R. M, Hurlbut, H S, Work, T. H, Kingston, J R, and Frothingham, T E. Sindbis Virus A Newly Recognized Arthropod-transmitted Virus *Am J Trop Med*, 4 844–862, 1955

59. Morgan, I M Quantitative Study of the Neutralization of Western Equine Encephalomyelitis Virus by Its Antiserum and the Effect of Complement *J Immunol*, 50 359–371, 1945
60 Whitman, L The Neutralization of Western Equine Encephalomyelitis Virus by Human Convalescent Serum The Influence of Heat Labile Substances in Serum on the Neutralization Index *J Immunol*, 56 97–108, 1947
61 Hammon, W McD Reports to Neurotropic Virus Diseases Commission, Army Epidemiological Board Unpublished, 1945–1953
62 Olitsky, P K, and Casals, J The Effect of Incubation at 37° C. on the Neutralization Test with Various Encephalitis Viruses Including Lansing Strains of Poliomyelitis Virus *J Immunol*, 60 487–496, 1948
63 Dozois, T F, Wagner, J C, Chemerda C M, and Andrew, V. M The Influence of Certain Serum Factors on the Neutralization of Western Equine Encephalomyelitis Virus *J Immunol*, 62 319–331, 1949
64 Howitt, B F The Complement Fixation Reaction in Experimental Equine Encephalomyelitis, Lymphocytic Choriomeningitis and the St Louis Type of Encephalitis *J Immunol*, 33 235–250, 1937
65 Casals, J, and Palacios, R The Complement Fixation Test in the Diagnosis of Virus Infections of the Central Nervous System *J Exper Med*, 74 409–426, 1941
66 Olitsky, P K, and Casals J Neutralization Tests for Diagnosis of Human Viral Encephalitides *J A M A*, 134 1224–1228, 1947
67 Morgan, I M, and Olitsky, P K Immune Response of Mice to Active Virus and to Formalin-Inactivated Virus of Eastern Equine Encephalomyelitis *J Immunol*, 42 445–454, 1941
68 Meiklejohn G, Rust, E, and Brunetti, O Comparison of Diagnostic Methods for Western Equine and St Louis Encephalitis *J Immunol*, 62 293–300, 1949
69 Sather, G E, Miller, B J, and Hammon, W McD Comparative Study of Strains of St Louis Virus *Proc Soc Am Bact*, 1953, p 49
70 Lennette, E H, Nyberg M C, Barghausen, D M, Chin, R, Fujimoto, F Y, and Itatani, M K The 1952 Outbreak of Encephalitis in California Laboratory Methods for Etiologic Diagnosis *California Med*, 79 78–83, 1953
*71 España, C, and Hammon, W McD An Improved Benzene Extracted Complement Fixing Antigen Applied to the Diagnosis of the Arthropod Borne Virus Encephalitides *J Immunol* 59 31–44 1948
72 Casals, J Acetone-Ether Extracted Antigens for Complement Fixation with Certain Neurotropic Viruses *Proc Soc Exper Biol and Med*, 70 339–343, 1949
73 Smadel, J E, Baird R D, and Wall M J Complement Fixation in Infections with the Virus of Lymphocytic Choriomeningitis *Proc Soc Exper Biol and Med*, 40 71–73, 1939
73a Whitney, Elinor, Kraft L M, Lawson W B, and Gordon I Noninfectious Complement-Fixing Antigen from Embryonated Hens Eggs Infected with Lymphocytic Choriomeningitis Virus *Proc Soc Am Bact*, 1953, p 50
74 DeBoer, C J, and Cox, H R Specific Complement Fixing Diagnostic Antigens for Neurotropic Virus Diseases *J Immunol* 55 193–204, 1947
75 Casals, J Ultraviolet Light-Inactivated Antigens for Complement Fixation Tests with Central Nervous System Virus Infections *Proc Soc Exper Biol and Med*, 49 501–504, 1942
76 Smadel, J E Personal communication, 1947
77 Havens, W P, Jr, Watson, D W, Green, R H, Lavin, G I and Smadel, J E Complement Fixation with the Neurotropic Viruses *J Exper Med*, 77 139–153, 1943.
78 Casals, J Complement Fixation Test with Encephalitis Viruses *J Immunol* 56 337–341, 1947
79. Hammon, W McD., and España, C Simple Method of Producing Control Guinea Pig Immune Sera for Use with Complement-Fixing Antigens for the Arthropod borne Virus Encephalitides *Proc Soc Exper Biol and Med.*, 66 113–115, 1947

43a Hammon, W. McD, and Sather, G E Immunity of Hamsters to West Nile and Murray Valley Viruses Following Immunization with St Louis and Japanese B *Proc Soc Exper. Biol and Med* March, 1956.

44 Olitsky, P K, Sabin, A B, and Cox, H R An Acquired Resistance of Growing Animals to Certain Neurotropic Viruses in the Absence of Humoral Antibodies or Previous Exposure to Infection *J Exper Med*, 64 723–737, 1936

45 Sabin, A B, and Olitsky, P. K Influence of Host Factors on Neuroinvasiveness of Vesicular Stomatitis Virus I Effect of Age on the Invasion of the Brain by Virus Instilled in the Nose. *J Exper. Med*, 66 15–34, 1937. III Effect of Age and Pathway of Infection on the Character and Localization of Lesions in the Central Nervous System *J Exper Med*, 67 201–228, 1938

46 Olitsky, P K, and Harford, C G Intraperitoneal and Intracerebral Routes in Serum Protection Tests with the Virus of Equine Encephalomyelitis I A Comparison of the Two Routes in Protection Tests *J Exper Med*, 68 173–189, 1938

47 Lennette, E H, and Koprowski, H Neutralization Tests with Certain Neurotropic Viruses, Comparison of Sensitivity of Extraneural and Intracerebral Routes of Inoculation for Detection of Antibodies *J Immunol*, 49 375–385, 1944

48 Beveridge, W I B, and Burnet, F M The Cultivation of Viruses and Rickettsiae in the Chick Embryo *Medical Research Council, Special Report Series, No 256*, 1946 His Majesty's Stationery Office, London

49 Reed, L J, and Muench, H Simple Method of Estimating Fifty Per Cent End-points *Am J Hyg*, 27 493–497, 1938

50 Pond, W L, Russ, Sudie B, Rogers, Nancy G, and Smadel, J E Murray Valley Encephalitis Virus, Its Serological Relationship to the Japanese-West Nile-St Louis Encephalitis Group of Viruses *J Immunol*, 75 78–84, July, 1955

51. Casals, J, and Olitsky, P K Enduring Immunity Following Vaccination of Mice with Formalin-Inactivated Virus of Russian Spring-Summer (Far Eastern, Tick-borne) Encephalitis *J Exper Med*, 82 431–433, 1945

52 Casals, J, and Brown, L V Hemagglutination with Certain Arthropod-Borne Viruses *Proc Soc Exper Biol and Med*, 83 170–173, 1953.

52a *Casals, J, and Brown, L. V Hemagglutination with Arthropod-Borne Viruses J. Exper Med*, 99 429–449, 1954

53 Clarke, D H, and Casals, J Improved Methods for Hemagglutination Studies with Arthropod-borne Viruses. *Proc Soc Exper Biol and Med*, 88 96–99, 1955

54 Personal communication, staff of the Rockefeller Foundation Virus Laboratories

55 Sabin, A B, and Buescher, E L Unique Physico-Chemical Properties in Japanese B Encephalitis Virus Hemagglutinin *Proc Soc Exper Biol and Med*, 74 222–230, 1950

56 Sabin, A B Hemagglutination by Viruses Affecting the Human Nervous System *Fed Proc*, 10 573–578, 1951

57 Chanock, R M, and Sabin, A B Hemagglutinins of St Louis and Western Equine Viruses Isolation, Properties, and Use for Diagnosis and Epidemiologic Survey *Am J Dis Child*, 84 628–629, 1952

58 Chanock, R M, and Sabin, A B The Hemagglutinin of St Louis Encephalitis Virus I Recovery of Stable Hemagglutinin from the Brains of Infected Mice II Physico-Chemical Properties and Nature of Its Reaction with Erythrocytes III Properties of Normal Inhibitors and Specific Antibody, Use of Hemagglutination-Inhibition for Diagnosis of Infection *J Immunol*, 70 271–316, 1953

58a Chanock, R M, and Sabin, A B The Hemagglutinin of Western Equine Encephalitis Virus Recovery, Properties and Use for Diagnosis *J Immunol*, 73 337–351, 1954

58b Chanock, R M, and Sabin, A B The Hemagglutinin of West Nile Virus Recovery Properties, and Antigenic Relationships *J Immunol*, 73 352–362, 1954.

58c Hammon, W. McD *Encephalitis: Eastern and Western Equine and St. Louis Types, as Observed in 1941 in Washington, Arizona, New Mexico and Texas. J.A.M.A.*, 121 560–566, 1943

RABIES

HARALD N. JOHNSON, M. D.

The Rockefeller Foundation Virus Laboratories
New York, N Y.

80 Fulton, F, and Dumbell, K R The Serological Comparison of Strains of Influenza Virus *J Gen Microb*, 3 97–111, 1949

81 Svedmyr, A, Enders, J F, and Holloway, A Complement Fixation with Brunhilde and Lansing Poliomyelitis Viruses Propagated in Tissue Culture. *Proc Soc Exper Biol and Med*, 79 296–300, 1952

82 Kraft, L M, and Melnick, J L Immunological Reactions of the Coxsackie Viruses II The Complement Fixation Test *J Exper Med*, 92 483–497, 1950

83 Bukantz, S C, Rein, C R, and Kent, J F. Studies in Complement Fixation II Preservation of Sheep's Blood in Citrate Dextrose Mixtures (Modified Alsever's Solution) for Use in the Complement Fixation Reaction *J Lab and Clin Med*, 31 394–399, 1946

84 Mayer, M M, Osler, A G, Bier, O G, and Heidelberger, M The Activating Effect of Magnesium and Other Cations on the Hemolytic Function of Complement *J Exper Med*, 84 535–548, 1946

85 Bedson, S P, and Bland, J O W Complement Fixation with Filterable Viruses and Their Antisera *Brit J Exper Path*, 10 393–404, 1929

along neuronal fibers When infection occurs, the disease develops most often between 4 and 6 weeks after exposure, but the incubation period may vary from 10 days to at least 8 months.

B. The Animal Reservoir of Rabies

There are two epidemiologic types of rabies—an urban type, which is propagated by domestic dogs, and a sylvatic type, which is maintained in wild animals Rabies in wild animals is likely to go unrecognized unless the disease occurs in epizootic proportions in wolves, foxes, coyotes, jackals, skunks, mongooses, or meerkats, and these animals attack and infect man and his domestic animals

The vampire bat, ***Desmodus rotundus murinus*** Wagner, is the only known host that can act as a carrier of rabies over an extended period without exhibiting symptoms of illness [1] These animals live on blood alone and feed by biting so that they do not need to become vicious in order to transmit the infection. They do spread the disease among themselves and to other species of bats when fighting. Vampire bats are found only in Mexico, Central America, and South America The occurrence of epizootics of cattle rabies in Brazil, Trinidad, and Mexico, in regions where dog rabies was rare or unknown, stimulated research on rabies which resulted in the discovery that rabies exists as an enzootic disease of vampire bats in these countries [2-5] Noncolonial, that is, free-living insectivorous bats of the ***Dasypterus floridanus*** species and the ***Lasiurus seminola*** species have been found infected with rabies in Florida, and a bat of the ***Lasiurus cinereus*** species has been found infected with rabies in Pennsylvania [6-7] It is possible that such species of migratory bats become infected with rabies by contact with vampire bats in Mexico or in Central America It is also possible that rabies may be present as an enzootic disease of insectivorous bats in some parts of the United States The free-living bats rest in bushes or trees and are taken as food by some carnivora, such as skunks, raccoons, and wildcats It is likely that the bats occasionally manage to bite the predator before they are killed, thus infected bats could spread rabies to the wild carnivora The dissemination of rabies by insectivorous bats would best account for the recent numerous concomitant outbreaks of skunk rabies in widely separated regions of central and western United States.

Though the bat family forms one potential reservoir of wild-life rabies, there may be other small mammals that can maintain the disease as a symptomless infection The marked susceptibility of the hamster

III. Biologic and Immunologic Procedures for Diagnosis
- A Choice of Animal for Test Inoculation
- B Preparation of Specimens for the Biologic Test
- C The Mouse Inoculation Test
- D Observation of Inoculated Mice
- E. Harvesting Mouse-Brain Specimens
- F The Microscopic Specificity Test
- G Passage and Titration of the Virus
- H Preservation of Rabies Virus
- I Pathogens Other than Rabies Virus to Be Ruled Out
- J The Serum-Virus Neutralization Test
  - 1 Immunologic specificity tests for rabies virus
  - 2 Preparation and storage of sera
  - 3 Preliminary titration of the unknown virus
  - 4 Stock virus for the serum-virus neutralization test
  - 5 Preparation of serum-virus mixtures
  - 6 Testing of serum-virus mixtures
  - 7 Observing inoculated mice and recording results
  - 8 Interpretation of the serum-virus neutralization test
  - 9 Dilutions of serum versus constant amount of virus
- K The Cross-Protection Test
- L The Complement Fixation Test

IV References

I. INTRODUCTION

A General Statement of the Disease

Rabies is a virus disease of animals which is transmitted from animal to animal, and animal to man, by biting. Infected domesticated dogs and cats are the common source of human infection. In these animals the disease often begins with such symptoms of excitation of the central nervous system as irritability and viciousness During the early

5 days of the onset of symptoms Some rabid dogs die during the excitation stage, but most of them will develop progressive inco-ordination and paralysis until prostrate and comatose before they die of the disease

In addition to infecting the central nervous system, the virus also may invade and multiply in the salivary glands Thus, the virus is excreted with the saliva, so that the bite of the vicious rabid animal introduces the virus into a fresh wound Under favorable conditions, the virus becomes established in the central nervous system by propagation

not uniformly invaded by the virus in rabid animals, in order to know if a person bitten by a rabid dog was actually exposed to the virus, the submaxillary salivary glands as well as the brain should be tested; of the salivary glands, the submaxillary glands are the best source of rabies virus Since these tests are time-consuming, however, they should not be used as a basis for the decision regarding vaccination.

Those who wish a guide for the handling of cases of animal bite are referred to the Report on the Second Session, WHO Expert Committee on Rabies [11]

D Recognition of Human Rabies

A correct clinical diagnosis of rabies in man usually will be made if symptoms of encephalitis develop 10 days or more after a bite by a dog or other animal, and if the physician is aware of the possibility of exposure to rabies The disease begins ordinarily with the symptoms headache, malaise, and low-grade fever and signs of excitation of the central nervous system such as irritability, constant volubility, apprehension, emotional instability, insomnia, and increased reflex activity of the musculature The excitation phase may progress until death occurs during a convulsive seizure More often, symptoms of depression of the nervous system develop before the patient dies In patients who exhibit marked excitation of the central nervous system, the disease is apt to run a rapid course, with a fatal outcome within 3 to 5 days of its onset Sometimes the disease is characterized by progressive ascending paralysis without a prodromal excitation phase, in such cases the disease is apt to be prolonged to a week or more The clinical picture is then similar to that of poliomyelitis and other conditions associated with paralysis

If a history of bite is not elicited, the possibility of rabies might not be considered unless the patient exhibits the classical hydrophobia symptom of episodes of painful spasmodic contractions of the throat muscles when attempting to swallow The hydrophobia symptom is by no means a constant feature of rabies in man, and this symptom has not been observed at all in patients that develop paralytic rabies after infection by vampire bat bite Difficulty in swallowing is a common feature of rabies in man, but until attempts to swallow precipitate painful contractions of the throat muscles, the patient will not develop a fear of water Sensory disturbances related to the site of exposure, such as shooting pains, burning, or tingling, indicate a diagnosis of rabies, but the association with rabies may be missed unless it is known that the patient was bitten by an animal

E Rabies and Paralysis from Vaccination

A person bitten by an animal ordinarily is given the rabies vaccine treatment if the biting animal is suspected of having rabies Therefore, if the patient develops paralysis in the course of, or soon after, the rabies vaccine treatment, the question arises whether the disease is paralytic rabies or paralysis from vaccination caused by sensitization to the brain tissue in the vaccine In the past, if the patient recovered, it was

to rabies virus infection when inoculated by the extraneural route and the occurrence of nonfatal infections in this host with some varieties of rabies virus warrant consideration of this and other small mammals as potential carriers of rabies.[8-9] The epidemiology of rabies in wolves and foxes in the arctic suggests that the disease may be present in small mammals such as lemmings, voles, or ground squirrels in this region and that the carnivorous animals become infected by bites of these animals.

In the United States rabies is present in enzootic proportions in foxes in most of the states east of the Mississippi and in Texas. Skunk rabies is present in the North Central and the Mountain-Pacific states. In Puerto Rico the mongoose is the wildlife vector of rabies.[10]

C. Dogbite and Prevention of Rabies after Exposure

Knowledge as to when exposure to rabies occurred makes it possible by vaccination to immunize and protect a high percentage of exposed individuals during the relatively long incubation period of the disease The administration of rabies hyperimmune serum offers an added safeguard by giving temporary protection. The serum alone may not prevent the disease in each case but it at least increases the incubation period sufficiently to allow for development of active immunity as a result of vaccination.

The frequency with which people are bitten by dogs makes it impossible to give the rabies vaccine treatment to all cases of dogbite exposure, even in regions where the disease is known to be prevalent Therefore, it becomes important to have a method of assessing the probability of exposure.

The question of whether or not exposure to rabies has occurred must be determined by what happens to the biting animal. For instance, if a dog that has bitten someone can be restrained and kept under observation for 7 days and the animal remains healthy, one can safely conclude that exposure to rabies has not occurred If the dog shows symptoms of rabies and then dies, it is desirable to examine the brain for Negri bodies because if these are found one can make a definite diagnosis of rabies. This should also be done with a dog that dies or is killed immediately or soon after biting If Negri bodies cannot be found, the decision regarding vaccination should depend on the history of the animal's past behavior The brain of the animal can be tested for the presence of rabies virus by the mouse inoculation test when the laboratory examination for Negri bodies is negative. But because the salivary glands are

antibiotics, such as penicillin and streptomycin, saliva specimens when introduced into the brain of such animals invariably produced bacterial sepsis Inoculation of the animals by the peripheral route was the only means of testing for the virus in saliva, and the low infection rate and the long incubation period of the disease in animals inoculated by this route made this a tedious and uncertain method of diagnosis It is possible to test saliva specimens for rabies virus by intracerebral inoculation into mice if this material is diluted in physiologic salt solution containing penicillin and streptomycin. This saliva test should be done on all patients that become ill after animal bite and in cases of encephalitis of unknown etiology

G Special Characteristics of the Virus

Rabies is one of the larger viruses and it is not readily filtrable. It will pass through diatomaceous earth and unglazed porcelain filters that withhold common varieties of bacteria, but it is retained by Seitz EK type, serum-sterilizing filter pads Rabies virus as found in infected brain or salivary gland tissue may remain infective for several weeks at the ordinary refrigerator temperature of 4° C if the tissue is free of bacteria and is protected from desiccation The virus may remain active for several weeks at room temperature, for months at 4° C, and for years at −12° C, if blocks of infected tissue are stored in undiluted neutral glycerol The extraction of water from the tissue by the glycerol may be equivalent to that obtained by freeze-drying. For instance, changing the glycerol once or twice will remove the water extracted from the tissue. The glycerol inhibits enzyme activity and suppresses bacterial organisms.

The resistance of rabies virus to heat depends on the physical state in which the virus is found For example, the virus as obtained in infected tissue, macerated in physiologic salt solution, is inactivated by exposure to a temperature of 56° C for 1 hour The desiccated tissue virus preserved in sealed glass ampules under an atmosphere of nitrogen may be exposed to the same temperature for an equal period of time with little, if any, demonstrable loss in infectivity.

H. Distribution of Virus in Animals

Rabies virus ordinarily is demonstrable in the central nervous system of man and animals dying of rabies. The medulla and thalamus usually contain the greatest concentration of virus, but the virus is present also in the cerebral cortex, cerebellar cortex, and the spinal

assumed that the disease could not be rabies because physicians were taught that no one recovered from this disease. For example, in Trinidad, vampire bat rabies, which produces only the paralytic type of rabies in man, was diagnosed as poliomyelitis until rabies virus was isolated from one case by inoculation of brain tissue into monkeys [12,13] Thereafter, a considerable number of similar cases were diagnosed in retrospect as paralytic rabies, where investigations of deaths that had occurred as a result of a paralytic disease showed a history of the deceased person's having been bitten by a vampire bat [14]

The general pathology produced by rabies and paralysis from vaccination is not sufficiently characteristic to allow a certain differential diagnosis Serologic studies will not aid in differentiating the two conditions because both are associated with an increase in rabies virus neutralizing substance in the blood serum during the course of the disease

The demonstration of Negri bodies or isolation of the virus from fatal cases does identify the majority of rabies cases When the disease is of long duration, autosterilization, a well-known phenomenon in virus infections, may take place, so that it is then impossible to isolate rabies virus from the patient after death, even if rabies virus was the cause of the disease.

The demonstration of rabies virus in the saliva is the only practical method of identifying nonfatal cases of rabies It is possible that the virus may be found in the spinal fluid, but this body fluid has not been found to contain the virus in the limited number of cases tested [15]

F. Necessity for Testing Saliva Specimens

The concept that rabies in man is always fatal is based on the knowledge that rabies virus has not been isolated from a human being that developed encephalitis and recovered The laboratory diagnosis of rabies, however, depends on the isolation of the virus from the saliva, and the testing of saliva for rabies virus has been done so rarely that there are only a few records of isolation of the virus from this source.[16] Of course, a negative saliva test does not rule out rabies infection since the virus may fail to become established in the salivary glands. There are practical reasons why the testing of saliva for rabies virus has been neglected in the past. It is essential to inoculate rabies virus into the brain of the common laboratory animals in order to be certain of producing a characteristic fatal infection. Before the discovery of modern

pigs are fairly susceptible to street rabies virus given by intramuscular inoculation, and these animals are useful for cross-immunization experiments and for testing the potency of rabies vaccine [18]

The age of the animal may influence the susceptibility to rabies. For example, high egg passage (HEP), Flury strain rabies virus, when inoculated into the brain tissue of infant mice, produces a fatal infection, but the same material given by the same route to mature mice produces no evident illness.[19]

The developing chick embryo is a suitable host for the cultivation of rabies virus.[9] However, inoculation of chick embryos as a test for the presence of street rabies virus is not recommended because infection is not produced consistently, and characteristic Negri bodies cannot be demonstrated in chick embryos infected with rabies virus Rabies virus can be cultivated in mouse embryo brain tissue culture [20,21] At the present time there is no practical tissue culture method for the isolation and identification of street rabies virus

K Virus Strains

Rabies virus strains, including those isolated from vampire bats, show cross complement fixation, cross serum-virus neutralization, and cross protection There are wide differences in the species pathogenicity of some natural varieties of rabies virus, notably that of vampire bat rabies virus and the *oulou fato* rabies virus. Rabies virus as it is found in naturally infected dogs can be altered by experimental methods so that it is harmless when given by subcutaneous and intramuscular injection to man and laboratory animals [19,22]

L Special Precautions for Workers

The Pasteur rabbit-brain-fixed rabies virus does not produce infection of the central nervous system in man when given by parenteral injection, as shown by the fact that vaccines containing active Pasteur vaccine-virus have been and still are used for the human rabies vaccine treatment The HEP strain of Flury chick-embryo-adapted rabies virus has been tested in man, and it produced no significant reaction when given by intramuscular injection [22] Therefore, there is no danger of infection with the Pasteur rabbit-brain-fixed rabies virus nor the HEP Flury chick-embryo-adapted rabies virus and no need for special vaccine treatment if a laboratory worker is exposed by accidental inoculation with these virus strains.

cord[15] As with other types of viral encephalitis, autosterilization may take place; that is, the virus may be neutralized by antibodies or inactivated virus

The transmission of rabies in nature depends on the ability of the virus to infect the salivary glands of a rabid animal, and these glands may contain more active virus per gram of tissue than the brain. About 75 per cent of naturally infected rabid dogs will have rabies virus in the submaxillary salivary glands Rabies virus seldom invades the parotid glands of dogs The virus may be found in the pancreas, in the lacrimal and adrenal glands, and in various parts of the nervous system, including ganglia and nerve trunks.[17]

Rabies virus, adapted to brain tissue by prolonged serial intracerebral passage in an experimental host, such as the Pasteur rabbit-brain-fixed virus, does not invade and multiply in the salivary glands The tropism of rabies virus for the salivary glands is reduced markedly by a few serial intracerebral passages of the virus in mice. Rabies virus has not been demonstrated in the blood, spleen, liver, lymph nodes, bone marrow, or sex glands of dogs infected experimentally with street rabies virus[17]

I Pathologic Lesions

The pathology produced in the central nervous system by infection with rabies virus is similar to that found in other types of viral encephalitis Unless Negri bodies are present, it is impossible to make a certain diagnosis of rabies by microscopic examination of the brain. Neuronal degeneration and perivascular cellular infiltration become more marked as the disease progresses When death occurs soon after the onset of symptoms, there may be no significant pathologic tissue changes in the brain.

The submaxillary salivary glands if infected with rabies virus show degeneration of the acinar epithelium and mononuclear cell infiltration of the interstitial tissue

J Susceptibility of Different Species

All of the common laboratory animals are susceptible to street rabies virus if it is inoculated into the brain. The white laboratory mouse is the most suitable animal for testing for the presence of rabies virus in tissue suspensions. Young hamsters are particularly useful for rabies studies which require extraneural exposure because in this species the intramuscular LD_{50} and intracerebral LD_{50} are about the same.[8] Guinea

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having rabies, are subject to exposure to rabies, and such persons should be immunized in the same manner as laboratory personnel

White mice used for virus isolation studies must be handled with forceps (this is standard procedure for all handling of live mice) Larger animals are handled with thick leather gloves. A convenient method of holding a dog is to grasp the upper jaw of the animal with the thumb on top and the fingers in the cleft of the lower jaw The other hand may be used to grasp the back of its neck If the dog is grasped by the lower jaw with the gloved hand so that the skin of its lips is pulled over the lower jaw teeth, the dog will not bite down on the gloved hand It is often necessary to muzzle dogs suspected of having rabies, this may be done with 2-inch gauze bandage by winding it about the jaws and crossing and tying the ends around the dog's neck

The use of surgeon-type rubber gloves is not recommended for laboratory workers Those persons engaged in opening containers and removing the brains and salivary glands from animal heads should wear heavy-duty industrial type rubber gloves

II METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

A Clinical Sources of Material for Testing

1. *Saliva specimens* Unless and until the patient dies, the saliva is the most likely source of virus in rabies cases The saliva specimen should be taken from under the tongue where the ducts of the submaxillary salivary glands open into the mouth If the patient is co-operative, this may be done with an insulin syringe or eye-dropper pipette. The saliva specimen then may be transferred to a rubber-stoppered or screw-top vial or a pyrex test tube For shipment it is essential to use a pyrex test tube and to glass-seal this with a torch The acetylene torch without added oxygen is excellent for sealing pyrex test tubes If the patient is excited, it may be necessary to obtain the saliva specimen as sputum in a paper container or glass petri dish If the sputum is scanty and viscid it may be diluted with physiologic salt solution so that the specimen can be transferred to a test tube In some cases the only way to obtain a specimen is to collect the saliva with a cotton swab on a wooden applicator The saliva is removed from the swab by agitation in a small amount of sterile physiologic salt solution, and this is saved as the saliva specimen

In laboratories where street rabies virus is handled in the form of specimens from cases of natural infection in man and animals or for experimental studies, workers who open containers that hold animal specimens, remove brain and salivary gland specimens, prepare brain tissue impressions and smears on glass slides and tissue suspensions, perform animal inoculation, or process tissue specimens from experimentally infected animals should be given a course of 7 injections of rabies vaccine at least 1 month before they are allowed to take part in such work. One or 2 doses of vaccine given annually thereafter should be sufficient to maintain an adequate level of immunity. As a guide in deciding whether revaccination is necessary, blood serum may be tested for antibodies to rabies virus, at yearly intervals. For example, if the neutralization test reveals neutralizing antibody for rabies virus in the blood serum, it is not necessary to give a booster dose of vaccine Immunity may be present even though the neutralization test method may fail to show a signficant level of virus-neutralizing antibody In experimental studies of resistance to infection with rabies virus in dogs vaccinated with killed-virus rabies vaccine when the animals were challenged by intramuscular injection of street rabies virus 1 year after vaccination, the neutralization test method failed to show a significant amount of virus-neutralizing antibody in the blood of about 50 per cent of the animals that resisted infection[23] Rabies hyperimmune serum is available commercially and should be kept on hand in laboratories processing tissue specimens infected with street rabies virus. The rabies-immune serum should be given promptly in cases of accidental inoculation exposure with street rabies virus The local treatment of wounds produced by instruments or glassware contaminated with street rabies virus is the same as for animal bites, that is, immediate thorough cleansing with soap or detergent solution The use of cautery with mineral acid is limited to treatment of puncture wounds which cannot be cleansed with soap or detergent solution Contamination of the unbroken skin with street rabies virus will not result in infection If the virus is spilled or splashed onto the skin, however, it is advisable to immediately wash with soap and water or a detergent solution and then soak or sponge the skin with bichloride of mercury solution at a strength of 0 02 gm per cent In addition to a stock bottle of bichloride of mercury the laboratory should have available a small bottle of nitric acid for use in cauterizing puncture wounds contaminated with street rabies virus. The acid treatment ordinarily is given by dipping the pointed end of a broken wooden applicator into the acid and by introducing this into the depth of the puncture wound After a 5-minute exposure to the acid the wound area is washed with soap and water and then covered with a paste of sodium bicarbonate and water to neutralize the remaining acid Exposure of the mucous membranes to street rabies virus is to be treated in the same manner as inoculation exposure[11] Pipettes used for mixing street rabies virus must be plugged with cotton If the cotton becomes moistened with the virus suspension the pipette should be discarded There is a particular hazard of exposure of the mucous membrane of the eye when opening the calvarium of animal heads. Goggles should be worn for this type of work.

Veterinary medical doctors and animal attendants engaged in rabies control work, such as observation and handling of dogs suspected of

animal lives, hence the practice of holding animals suspected of having rabies until they die. It is advisable also to examine specimens from all animals suspected of having rabies in order to have definite information as to the presence and extent of the disease.

D. Packing and Shipping of Animal Heads

When it is necessary to ship an animal head to a public health laboratory for diagnosis, the head must be removed by severing near the shoulders so as to leave the salivary glands unexposed. Then the specimen should be placed in a water-tight metal container and this, in turn, in a larger container of the same type, of sufficient size to permit adequate packing with water ice and sawdust. If a messenger service is not available for delivery to the laboratory, the shipment must be made by express freight. Carbon dioxide ice must not be used for refrigeration of animal heads because the freezing alters the tissue in such a way that a satisfactory microscopic examination cannot be made.

E. Shipment of Spinal Fluid, Saliva, or Tissue Specimens

Spinal fluid and saliva specimens that must be shipped to a diagnostic laboratory for virus isolation studies should be put into pyrex glass test tubes, flame-sealed, and packed in a metal mailing container such as those used for bacteriologic specimens. This container is then placed in a thermos bottle containing carbon dioxide ice or packed in a carton with carbon dioxide ice for shipment by express freight. If microscopic examination has been completed and only virus isolation studies are desired, tissue specimens are preserved best by shipment in flame-sealed pyrex glass tubes as recommended for spinal fluid and saliva specimens. The tissue specimens may be shipped without refrigeration if small blocks of tissue are placed in undiluted neutral glycerol in a strong glass vial and sealed with a sleeve-type rubber stopper or some other type of rubber stopper that can be secured in place with tape. If the vial is then packed in a double metal container, such as those used for bacteriologic specimens, it may be shipped by mail.

F. Handling of Animal Head Specimens in the Laboratory

When an animal head specimen [illegible]

face contamination. If the brain is removed first, one cannot be sure

2. *Spinal fluid specimens.* If a patient exhibits signs of encephalitis, a spinal fluid specimen ordinarily is obtained for study to rule out bacterial infections and for cell count and chemistry studies. Though there is little chance of isolating rabies virus from the spinal fluid, this material should be tested for viruses by the mouse inoculation test in both infant and mature mice.

3 *Other specimens for virus isolation studies.* It may be of interest to test the nasal and lachrymal secretions for rabies virus by the mouse inoculation test. The virus may be found in the lachrymal glands[15]

B. Post-Mortem Sources of Material for Testing

1. *Brain specimens* Rabies is a disease of the central nervous system, and the infectious agent is always present in the brain tissue early in the course of the disease Except for rare instances of autosterilization where the virus is masked by antibodies or dead virus, it is possible to demonstrate rabies virus in the brain tissue of man and animals that die of the disease Blocks of tissue about 1 cm square should be taken from the medulla, pons, hippocampus, thalamus, and cerebral cortex and pooled for virus studies Similar portions of the Ammon's horn of the hippocampus, the cortex of the frontal and parietal lobes of the cerebrum, and the cortex of the cerebellum should be taken for examination for Negri bodies The medulla, pons, and thalamus are valuable for the demonstration of inflammatory lesions, and these should be included with the specimens saved for preparation of paraffin sections.

2. *Salivary gland specimens* Though the virus must invade the salivary glands in order to maintain the disease, infection of the salivary glands does not occur in all cases of rabies[15,17] The submaxillary salivary glands are the best source of rabies virus from tissues other than that of the central nervous system, and these glands may contain more virus per gram of tissue than the brain.

C. Animal Specimens

The diagnosis of rabies in animals is a necessary function of public health laboratories wherever the disease exists It is essential to have some method of ascertaining the risk of rabies exposure in dogbite cases; this is done by confinement and observation of biting dogs and examination of the brains of animals that die or are killed at the time of biting or during the period of quarantine The probability of finding Negri bodies in the brain of the rabid animal increases the longer the

a clean glass slide between the thumb and forefinger apply the slide gently against the exposed cut surface of the tissue Remove quickly This leaves a thin film of tissue on the slide—a mirror image of the cross section Make 2 or 3 impressions on the same slide Impressions of the cerebellar and cerebral cortex are prepared in the same manner.

For smear preparations cut out a portion of tissue about 1 mm in diameter from the Ammon's horn or other tissue and place this near one end of a glass slide Superimpose a 2d slide on the 1st, flatten the tissue by gentle pressure, and then draw the top slide lengthwise over the other, leaving a thin elongated smear on both slides

I Staining of Impressions and Smears

The wet impressions or smears of brain tissue are fixed and stained at the same time by immersion in Sellers' stain[24] This staining procedure is a modification of van Gieson's stain and is composed of a mixture of basic fuchsin and methylene blue. Each stock stain is prepared as a 1 gm per cent solution in absolute, acetone-free, methyl alcohol The formula specifies basic fuchsin—color index No 677 or Schultz No 780, and methylene blue—color index No 922 or Schultz No 1038 Store the stock stains in glass-stoppered bottles

To prepare the working stain solution take 1 part of the basic fuchsin stock and 2 parts of the methylene blue stock stain solutions; for example, 25 ml of basic fuchsin solution and 50 ml of methylene blue solution Mix but do not filter It is convenient to keep the working stain in a Coplin staining jar with a screw-top cover The stain solution may be used as long as it produces the proper staining reaction

Immediately after preparing impression or smear preparations immerse them in the stain solution for 1 to 5 seconds, depending on the thickness of the smear, then rinse with gently running water under the tap, and air-dry The preparation is ready for examination The stain fixes and stains the tissue at the same time. Dry brain tissue impressions and smears fixed while still moist with absolute methyl alcohol are suitable for staining with Sellers' stain, but such preparations must be stained for 5 minutes They are not so satisfactory as those which are fixed and stained at the same time Tap water ordinarily contains electrolytes which improve the quality of the staining reaction as compared to distilled water In some regions, however, the tap water contains chemical substances which alter the staining reaction unfavorably Comparison of preparations rinsed with tap water and distilled water

that the salivary glands are not contaminated with virus obtained from the brain, therefore, the salivary glands must be removed first if they are to be tested for virus. When the skin is reflected from a ventral midline neck incision, the submaxillary salivary glands will be exposed at the angle of the jaw These are firm, well-demarcated, pale reddish brown, lobulated glands about 1 5 by 2 5 by 3.5 cm. in size. The salivary glands may be placed in a sterile petri dish for refrigeration, pending processing

For removing the brain from an animal head, the head is held firmly by grasping the lower jaw with a lion-jawed bone-holding forceps. The operator exposes the calvarium with a midline longitudinal incision, reflects the skin laterally, and cuts away the muscle tissue to the base of the ears. The skull is opened with a sharp butcher's cleaver or a bone saw by a cut across the occipital area, then laterally on each side, and finally transversely just behind the eyes The calvarium then is pried up from the front and broken backward, thereby exposing the brain. The meninges are reflected laterally The muzzle of the head is held upright, and the brain is removed without handling by cutting under the frontal lobes and finally severing the cranial nerves and brain stem, allowing the brain to fall onto a paraffinized paper plate or metal pan

G Source of Material for Microscopic Examination

Portions of the Ammon's horn of the hippocampus, the cerebellar cortex, and the cortex of the frontal and parietal lobes of the cerebrum should be examined for Negri bodies The hippocampus is exposed by placing the brain base down and by making a deep longitudinal incision, with scissors or knife, into one hemisphere; this is made from the occipital pole anteriorly just lateral and parallel to the superior longitudinal fissure This cut exposes the lateral ventricle, the hippocampus will be recognized as a glistening white cylindrical body bulging up from the floor of the posterior part of the lateral ventricle The Ammon's horn is identified by sectioning transversely to show the characteristic concentric light and dark zones

H Preparation of Impressions and Smears

With a pair of straight scissors, cut through the Ammon's horn transversely, removing a cross section about 2 mm. thick Place this on a piece of blotting paper or a wooden tongue depressor. Press the one cut surface gently onto the paper or wood to make it adhere. Holding

a clean glass slide between the thumb and forefinger apply the slide gently against the exposed cut surface of the tissue. Remove quickly. This leaves a thin film of tissue on the slide—a mirror image of the cross section Make 2 or 3 impressions on the same slide Impressions of the cerebellar and cerebral cortex are prepared in the same manner.

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containing M/150 phosphate buffer at pH 7.0 will show whether tap water is suitable for routine use.

J. Examination of Stained Tissue Preparations

The stained tissue should appear *reddish violet or purplish blue*, depending on the density of the tissue. Use the low-power, 16 mm objective, for preliminary observation Select areas showing numerous large neurons where there is little or no overlapping of cells and study with the oil immersion objective

In a properly stained preparation the cytoplasm of the neurons will be blue or purplish blue, the nuclei and nucleoli will be a deeper blue; the stroma will be rose pink, nerve fibers will be a deeper pink; neural sheaths do not stain, bacteria, if present, are stained an intense blue; and erythrocytes are a copper color. Negri bodies, the specific cytoplasmic inclusion bodies of rabies, are found in the cytoplasm of large neurons which present the ballooning type of degeneration The Negri body is a sharply defined, spherical, oval, or elongated body, ordinarily 2 to 10 μ in diameter Several inclusion bodies, usually of variable size, may be present in 1 neuron and are found most often in the cytoplasm between the nucleus and the dendritic prolongations of the cell They may be found in the first part of the dendrite, and in such instances they are elongated The larger Negri bodies contain blue staining granules or inner bodies, often arranged in concentric layers; and the ground substance of the inclusion body is finely granular, takes the fuchsin stain, and appears cherry red in preparations stained with Sellers' stain The blue staining granules in the Negri body are about 0.2 to 0.5 μ in diameter *Small cytoplasmic inclusion bodies may contain a single central* blue staining granule, but most of the small forms have no evident inner structure, therefore, in the absence of larger forms these cannot be regarded as specific for rabies. The characteristic Negri bodies are usually more abundant in the Ammon's horn of the hippocampus than elsewhere in the brain, but they may be found in large numbers in the pyramidal cell layer of the cerebral cortex and the Purkinje cell layer of the cerebellum They occur commonly in the cranial nerve nuclei, but it is difficult to obtain satisfactory impression or smear preparations from this part of the brain. In impression and smear preparations the Negri bodies often appear to be outside the neurons. The Negri bodies are found in swollen cells undergoing degeneration, which are ruptured easily. Intracellular location of Negri bodies need not be demonstrated because the

staining reaction with Sellers' stain is characteristic, and the specific type of cell inclusion may be recognized when it is outside as well as inside the cells. It is ordinarily possible to find Negri bodies in the meshwork of the cytoplasm of degenerating neurons.

Cytoplasmic inclusion bodies, caused by diseases other than rabies and which stain red with Sellers' stain, may be found in dog, cat, and mouse brains. These inclusion bodies do not contain any inner bodies or granules, and they are more refractile and homogeneous in appearance than is the Negri body This type of intracytoplasmic inclusion body is found in animals infected with distemper virus when it invades the central nervous system There are cytoplasmic secretory granules in the pons which resemble the cytoplasmic inclusion bodies caused by some viruses The secretory granules in the acinar epithelial cells of the submaxillary salivary glands may coalesce to form cytoplasmic fuchsin or eosin staining bodies of the same size as Negri bodies

K Preparation and Staining of Paraffin Sections

The paraffin section method is not recommended for routine diagnostic demonstration of Negri bodies in animal brains Post-mortem tissue material from human cases should be fixed in Zenker's fixative Add glacial acetic acid to a concentration of 5 per cent just before use Make impression and smear preparations also and stain these with Sellers' stain; a microscopic diagnosis may be made promptly by this method The Zenker's fixative does not penetrate the tissue very well; therefore the tissue blocks should not be more than 3 mm thick Fix the tissue for 24 hours; then wash thoroughly in running water and store in 80 per cent ethyl alcohol After embedding in paraffin, the sections must be treated with Lugol's solution and sodium thiosulfate solution to remove the corrosive sublimate crystals deposited in the tissue by the fixative. The phloxine and methylene blue stain and the Wolbach modification of Giemsa's stain, as given by Mallory, are suitable for the demonstration of Negri bodies in paraffin sections[25] Giemsa stain may be prepared, using a known amount of the azure dyes[28] In order to obtain uniform results, dilute Giemsa stain in M/150 phosphate buffer pH 7 0 to 7 5 and use the buffer solution for rinsing the slides Tap water may alter the staining reaction

The eosin and methylene blue staining technic used for routine histology in many pathology laboratories should be satisfactory for staining Negri bodies in tissue fixed in Zenker's solution This method may be improved by using the sodium

ethyl eosinate at a 1 per cent concentration in 95 per cent ethyl alcohol with the pH adjusted to 3 5 to 4 5 with acetic acid and staining in this eosin solution for 30 minutes Rinse and counterstain for 5 minutes in Unna's alkaline methylene blue stain[27] Use M/150 phosphate buffer at neutral pH for rinsing the stained sections Differentiate in absolute alcohol containing a small amount of rosin This method is applicable for staining smear and impression preparations fixed in Zenker's fixative, and such preparations are fixed adequately in 1 hour Mann's stain as given by Kraus, Gerlach, and Schweinburg is prepared by mixing 35 ml of a 1 per cent water solution of eosin with 35 ml of a 1 per cent water solution of methyl blue, and adding this to 100 ml of distilled water According to this method the sections are stained for 24 hours, then differentiated in absolute ethyl alcohol containing a small amount of sodium bicarbonate, and then exposed for a short time to a dilute solution of acetic acid in water Otherwise the sections are dehydrated and mounted in the usual manner[28] Note that this stain procedure calls for methyl blue and not methylene blue Tissue fixed in formalin or Zenker's fixative containing formalin at a concentration of 10 per cent and stained with hematoxylin and eosin may show some of the cytoplasmic inclusion bodies of rabies, but the inner structure is not stained so that this method is not applicable to the diagnosis of rabies

L INTERPRETATION OF OBSERVATIONS

The demonstration of Negri bodies in the brain makes it possible to make a definite diagnosis of rabies The frequency with which Negri bodies can be found in the brains of animals dying of rabies is illustrated best by citing the results of microscopic examination and animal inoculation obtained by different laboratories The studies of Negri–Luzzani as given by Koch list 4,961 brain specimens as positive for rabies by animal inoculation, and of these 6 7 per cent were negative for Negri bodies by microscopic examination[29] A comparison of the microscopic examination and the animal inoculation methods of diagnosis was made by Koch and Jahn for the period 1913 to 1928 Table 1 shows the results obtained as given by Koch[29]

Attention is called to the cyclical variation in the percentage of rabid animals showing Negri bodies—increasing with periods of high incidence and decreasing during periods of low incidence of rabies Table 2 shows the results of microscopic and animal inoculation tests of routine animal brain specimens submitted to the Georgia State Health Department during 1937.[30]

The average percentage of virus positive specimens missed by microscopic examination was 10.5 per cent A total of 236 specimens found positive by microscopic examination was also positive by animal inoculation

Dogs that develop furious rabies are the ones that bite people, and most of the specimens submitted to a diagnostic laboratory are from animals that have bitten someone. It is possible to make a definite diagnosis of rabies by microscopic examination in about 90 per cent of the dogs that develop furious rabies. Dogs that develop paralytic rabies

TABLE 1

SUMMARY OF MICROSCOPIC AND ANIMAL INOCULATION STUDIES OF ROUTINE BRAIN SPECIMENS RECEIVED AT THE ROBERT KOCH INSTITUTE

1913 to 1928

Year	Total Specimens	Negri +	Negri 0 Animal +	Negri 0 Animal 0	Per Cent of Total + Found Negri 0
1913	215	58	17	137	22.7
1914	120	37	7	68	15.9
1915	452	253	48	140	15.9
1916	538	283	21	226	6.9
1917	407	241	23	134	8.7
1918	405	235	28	132	10.7
1919	480	250	64	143	20.4
1920	355	123	45	170	26.8
1921	506	226	45	223	16.6
1922	706	373	58	258	13.4
1923	1,514	949	72	448	7.1
1924	1,700	868	73	733	7.7
1925	477	137	36	299	20.8
1926	283	72	14	193	16.2
1927	113	15	3	95	16.6
1928	95	7	3	85	30.0
Total	8,366	4,125	557	3,484	11.8

NOTE—It will be noted that columns 3, 4, and 5 do not add up to equal the total in column 2. Some of the Negri negative specimens were evidently unsatisfactory for animal inoculation because of decomposition and bacterial contamination.

without any signs of viciousness usually die within 3 days of the onset of symptoms, and of the dogs that develop this type of rabies only about 50 per cent show Negri bodies in the brain. In human rabies cases both the excited and paralytic forms of the disease are examined, and for all types of the disease about 70 per cent will show Negri bodies in the brain.

M. Reporting the Results

The finding of Negri bodies in a brain specimen is sufficient evidence for making a definite diagnosis of rabies The written or telegraphic report may be worded "The head submitted by you . ... 195.. is positive for rabies" Do not report a specimen as questionable positive or suspicious positive for rabies If not positive for Negri bodies, it must be called negative

TABLE 2

Summary of Microscopic and Mouse Inoculation Studies of Routine Brain Specimens Received at the Georgia State Health Department

1937

Month	Negri +	Negri 0	Negri 0 Mouse +	Total +	Per Cent of Total Positive Found Negri 0
January	62	37	5	67	7.5
February	50	38	1	51	2 0
March	67	48	13	80	16 2
April	76	48	6	82	7 3
May	77	93	7	84	8 3
June	58	76	9	67	13.4
July	73	68	8	81	9 9
August	39	63	11	50	22 0
September	48	39	4	52	7.7
October	45	35	6	51	11 8
November	53	45	7	60	11 7
December	42	33	4	46	8 7
Total	690	623	81	771	10 5

It is customary to report rabies positive specimens by telegraph or telephone, whether or not this has been requested The results of the microscopic examination must be reported by mail, and additional information and advice are included as needed for protection of persons and livestock exposed by a biting animal When Negri bodies are not found in a brain specimen, the report may be worded "The . . head submitted by you 195 . . is negative for Negri bodies This does not exclude the possibility of rabies The results of the mouse inoculation test will be reported later Consult your health officer or your physician and follow his advice" The laboratory report, whether positive or negative, offers an opportunity for distribution of educational literature on the subject of rabies This is the logical time to give out correct information on how to control rabies

III BIOLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS

A Choice of Animal for Test Inoculation

The white laboratory mouse is the best experimental host for the isolation and identification of rabies virus, and any of the various varieties of white mice are suitable for use as test animals[31] It is preferable to use Swiss albino mice of a genetic line that is known to be susceptible to a wide variety of virus diseases because virus diseases other than rabies may be encountered in routine biologic testing of human and animal tissue specimens There is at least one genetic variety of white mice which is resistant to 17D yellow fever vaccine virus and several viruses which have been isolated from arthropods, whereas other genetic types uniformly sicken and die after intracerebral inoculation with these viruses The variety of mice selected as test animals should be free of paratyphoid and other *Salmonella*, *Bartonella* and *Toxoplasma* parasites, and mouse encephalomyelitis, lymphocytic choriomeningitis, and ectromelia viruses The presence of enteric pathogens in the mice can be determined by testing a pool of fecal specimens from a box of young mice by bacteriologic culture methods used for the isolation of human enteric pathogens To test for the presence of mouse encephalomyelitis virus, collect a pool of fecal specimens from a box holding 25 or more recently weaned mice Grind this material in a mortar, in a diluent of physiologic salt solution or distilled water containing 500 units of penicillin and 1 mg of streptomycin per ml Sediment the bacterial component of the suspension by centrifugation in an ultracentrifuge for 20 minutes at 15,000 r p m or at maximum speed in an ordinary horizontal centrifuge for 1 hour Test the supernatant fluid for virus by injecting 0 03 ml intracerebrally into each of 10 mice of about 6 weeks of age If mouse encephalomyelitis is present as an enzootic disease in the stock mice, some of the inoculated mice will develop paralysis and die within 10 days after inoculation The incubation period may be as short as 4 days and as long as 28 days Make at least 1 blind passage using a brain pool from 3 mice sacrificed at 5 days after inoculation The chronic systemic infections of mice may be demonstrated by testing a pool of spleen tissue from 10 three-week-old mice Prepare a 10 per cent suspension of the spleen tissue in physiologic salt solution by grinding in a mortar. Do not add any antibiotic to the diluent and avoid

centrifugation Allow the suspension to stand at refrigerator temperature for about 30 minutes so that the particulate material will settle. The biologic test of the supernatant fluid is performed by intracerebral inoculation of 0 03 ml. into each of 10 mice approximately 6 weeks of age If the mice sicken, kill some of them with chloroform and take brain specimens for passage and for microscopic examination Make impression preparations on a glass slide from a cross section of the brain the same way as for rabies diagnosis In this case, however, fix the tissue with absolute methyl alcohol and stain with Giemsa's stain according to the method commonly used for the demonstration of malaria parasites[26] Parasites, bacteria, rickettsia, or elementary bodies if present will be revealed in the stained tissue

Mice of all ages are susceptible to infection with street rabies virus inoculated into the brain, but it is convenient to use 3- to 6-week-old mice for diagnostic work because the skulls are soft and the needle can easily be introduced into the parietal area. There are varieties of rabies virus, *such as the high-egg-passage (HEP) Flury vaccine virus*, which are pathogenic for infant mice but not for mature mice The HEP Flury virus does not produce clinical signs of infection in mature mice, dogs, or rabbits after intracerebral inoculation but produces paralysis and death in infant mice with an infectivity titer comparable to that obtained ordinarily with street rabies virus[19]

B. Preparation of Specimens for the Biologic Test

Spinal fluid specimens are suitable for testing without processing, but if the specimen consists of 5 ml or more of fluid, centrifuge this at medium speed in an ordinary centrifuge to sediment the cell component Resuspend the sediment in a quantity of the supernatant fluid sufficient for inoculation of the mice Rabies virus, if present in the spinal fluid *of experimentally infected dogs, is associated with the cellular* component[17]

Saliva specimens must be diluted in physiologic salt solution or distilled water containing 500 units of penicillin and 1 mg of streptomycin per ml It is advisable to test at least 2 dilutions of saliva, ordinarily 1:10 and 1 100 The addition of inactivated normal guinea pig or rabbit blood serum to the diluent to a concentration of 2 per cent is recommended if available, but rabies virus survives very well in the dilutions of saliva used for test inoculation The inoculation of undiluted saliva into the brains of mice is apt to kill a high proportion of the animals

To prepare brain or salivary gland specimens for the biologic test macerate the tissue in a diluent of physiologic salt solution to give a 10 per cent suspension of tissue by weight, that is, the weight of the tissue in grams multiplied by 9 gives the amount in ml of diluent to be used The common method of preparing a suspension of tissue for test inoculation is to grind it, using a mortar and pestle Use a pipette or syringe and needle to transfer the tissue suspension from the mortar to the test tube. Brain tissue may be ground into a fine suspension without the use of an abrasive, but salivary gland tissue must be minced with scissors and ground with sterile sand or alundum in order to obtain a fine suspension. It is not necessary to add serum to the diluent unless the tissue suspension is diluted more than 10^{-3} The frequency of bacterial contamination of routine animal specimens makes it advisable to add 500 units of penicillin and 1 mg. of streptomycin per ml of diluent Portions of the medulla, pons, hippocampus, thalamus, and cerebral cortex should be included in each brain pool to be tested. For testing salivary gland tissue take a 2 mm thick cross section from the center of each submaxillary salivary gland and prepare a 10 per cent suspension in physiologic salt solution from a pool of these 2 portions of tissue It is customary to centrifuge brain and salivary gland tissue suspensions at medium speed for 10 minutes to sediment the larger particles, which otherwise may clog the needle used for the inoculation procedure If tissue suspensions are allowed to stand for 1 hour, however, the larger particles will settle, and the supernatant fluid will be suitable for use in the mouse test At least 6 mice are needed for each specimen

C The Mouse Inoculation Test

The intracerebral test inoculation in mice is given by means of a 0 25 ml tuberculin syringe and a ½ inch, 26- or 27-gauge needle The dose per mouse is 0 03 ml for mature mice and 0 015 ml for infant mice No antiseptic is necessary or desirable over the inoculation site The mice may be anesthetized in a glass battery jar provided with a wire mesh platform to separate the mice from the ether-saturated cotton on the bottom of the jar The anesthetized mouse ordinarily is held on its side by grasping the head with the thumb and index finger of one hand The syringe can then be held by the other hand, with the index finger on the plunger and the barrel can be stabilized between the thumb and 3d and 4th fingers The forearm should rest on the table to stabilize the arm The needle should be introduced into the central part of the

upper parietal area, with the syringe kept as near horizontal as possible. It is advisable to inoculate the mice on a metal tray covered with a piece of heavy wrapping paper so that a clean working surface is available for testing each specimen Once a mouse is laid down on the working area, it is potentially contaminated and must not be put back into the ether jar The inoculation should be made into the cerebral hemisphere at a depth of about 1 mm below the surface of the brain. Place the inoculated mouse in a properly labeled box to the side of the operator and away from the hand holding the syringe in order to avoid the necessity of crossing the hands, because this might result in catching the fingers of one hand on the needle attached to the syringe held by the opposite hand. Bichloride of mercury solution 0.02 gm. per cent is a good disinfectant for inactivating rabies virus if any of the tissue suspension is spilled on the table or floor. It is not necessary to flame the top of the test tube before removing some of the tissue suspension, but the top of the tube must be flame-treated properly after such handling. The test tubes used for holding the tissue suspensions must be of pyrex, without lip. Since the inside of the test tube is contaminated, the syringe should not be put down inside the tube, rather, fill the syringe by tipping the tube and putting the needle tip just inside the opening to take up the fluid. As the hub of the needle will be held by the fingers this must not be put against the side of the tube It is convenient to have some sterile cotton in a jar on the work table, and if there is an air bubble in the syringe this may be expressed gently into a thick wad of cotton held over the point of the needle. The contaminated cotton may be put into the paper bag for burning or into a pan of water for sterilization in boiling water When the test inoculation is completed, the syringe and needle should be placed in an instrument pan for sterilization in boiling water. The syringe and needle may be rinsed with water provided that the needle is kept below the surface of the water in order to avoid producing an aerosol, which is dangerous if inhaled and which might contaminate the working area A small amount of detergent added to the water makes it easier to clean the instruments after sterilization in boiling water. The instruments must be subjected to boiling water for a minimum period of 5 minutes.

D. Observation of Inoculated Mice

If the specimen contains rabies virus, some of the mice inoculated with it usually show tremulous muscular activity, inco-ordination, or paralysis between 6 and 8 days after inoculation Other symptoms in-

clude humping of the back, ruffing of the fur, and conjunctivitis. Generalized convulsive seizures are common, and the animal may die during such a seizure Most of the mice infected with street rabies virus will develop flaccid paralysis of the legs The symptoms of paralysis ordinarily progress to complete prostration before death

For routine mouse inoculation tests it is customary to discard the surviving mice at the 28th day after inoculation Certain strains of street rabies virus are characterized by an incubation period of 15 to 20 days in mice inoculated intracerebrally and in rare instances the incubation period may be as long as 65 days [17]

Therefore, if the circumstances of the case indicate special handling, the mice may be held for 3 months In such cases it is advisable to retest the original specimen, using a larger group of mice including infant mice under 5 days of age in addition to mature mice

E Harvesting Mouse-Brain Specimens

To remove the brain from a dead mouse or one killed with chloroform after it develops symptoms of rabies, place the dead mouse on a small metal tray covered with a piece of paper towel or heavy wrapping paper. Wet the head with 70 per cent ethyl alcohol and expose the skull by reflecting the skin from the midline, using forceps and 115 mm. scissors with curved points With another pair of forceps and scissors cut around the calvarium and so expose and remove the brain Place the brain in a small petri dish

F The Microscopic Specificity Test

The microscopic examination of mouse brains for Negri bodies is necessary in order to establish a diagnosis of rabies Negri bodies will be found regularly in the brains of mice developing rabies after intracerebral inoculation with street rabies virus of canine origin Negri bodies are found more readily in mice which have been paralyzed for at least 24 hours Prepare an impression of a cross section of the mouse brain on a glass slide, taken so that it will include the hippocampus region of the brain Stain this while still moist with Sellers' stain according to the method described previously.

G Passage and Titration of the Virus

For passage prepare a 10 per cent suspension of mouse brain tissue from the infected mouse, using physiologic salt solution as a diluent Centrifuge for 10 minutes at 2,000 r p m ; save the supernatant fluid and use for passage. For storage and titration it is preferable to prepare

upper parietal area, with the syringe kept as near horizontal as possible. It is advisable to inoculate the mice on a metal tray covered with a piece of heavy wrapping paper so that a clean working surface is available for testing each specimen Once a mouse is laid down on the working area, it is potentially contaminated and must not be put back into the ether jar The inoculation should be made into the cerebral hemisphere at a depth of about 1 mm. below the surface of the brain. Place the inoculated mouse in a properly labeled box to the side of the operator and away from the hand holding the syringe in order to avoid the necessity of crossing the hands, because this might result in catching the fingers of one hand on the needle attached to the syringe held by the opposite hand Bichloride of mercury solution 0.02 gm per cent is a good disinfectant for inactivating rabies virus if any of the tissue suspension is spilled on the table or floor It is not necessary to flame the top of the test tube before removing some of the tissue suspension, but the top of the tube must be flame-treated properly after such handling The test tubes used for holding the tissue suspensions must be of pyrex, without lip Since the inside of the test tube is contaminated, the syringe should not be put down inside the tube; rather, fill the syringe by tipping the tube and putting the needle tip just inside the opening to take up the fluid. As the hub of the needle will be held by the fingers this must not be put against the side of the tube. It is convenient to have some sterile cotton in a jar on the work table, and if there is an air bubble in the syringe this may be expressed gently into a thick wad of cotton held over the point of the needle The contaminated cotton may be put into the paper bag for burning or into a pan of water for sterilization in boiling water. When the test inoculation is completed, the syringe and needle should be placed in an instrument pan for sterilization in boiling water. The syringe and needle may be rinsed with water *provided that the needle is kept below the surface of the water in order* to avoid producing an aerosol, which is dangerous if inhaled and which might contaminate the working area A small amount of detergent added to the water makes it easier to clean the instruments after sterilization in boiling water. The instruments must be subjected to boiling water for a minimum period of 5 minutes.

D Observation of Inoculated Mice

If the specimen contains rabies virus, some of the mice inoculated with it usually show tremulous muscular activity, inco-ordination, or paralysis between 6 and 8 days after inoculation. Other symptoms in-

rodent) may be present in brain specimens, and the symptomatology and incubation period of toxoplasmosis in mice inoculated intracerebrally with this parasite is similar to that produced by rabies virus

J. The Serum-Virus Neutralization Test

1. *Specificity tests for rabies virus* The neutralization test is not used for the routine diagnosis of rabies A definite diagnosis of rabies can be made by demonstrating Negri bodies in the brain of the naturally infected host that dies of the disease or in the brains of mice developing rabies after intracerebral inoculation with rabies virus derived from saliva or from brain or salivary gland specimens The neutralization test is used to check the identity of brain-fixed strains of rabies virus because Negri bodies are not produced in the brains of animals infected with such varieties of rabies virus The *oulou fato* variety of rabies virus found in Africa is a natural variant of rabies virus which has characteristics much like those of the brain-fixed strains, although the serum-virus neutralization test and the cross-protection test show that it is street rabies virus, it produces only the paralytic form of the disease, and Negri bodies are not found in the brains of animals infected with this virus [32]

The immunologic identification of a virus is accomplished by comparing it with a virus strain of known characteristics or an immune serum prepared by immunization of an animal with the Pasteur strain of rabies virus If the animals immunized with a known rabies virus are resistant to infection with an unknown virus, it is assumed that they are the same disease agent In order to identify an unknown virus by the neutralization test, titrate the virus in known normal and rabies-immune serum If the unknown virus is neutralized by the rabies-immune serum and not by the normal control serum, it is assumed to be rabies This is not so good a proof of identity as a cross-protection test, but it is a good method for the identification of rabies virus which is not known to be neutralized by immune serum prepared by immunization with viruses other than that of rabies However, it must be noted that neutralizing substance active against rabies virus may be found in the blood of dogs which have not been immunized and where there is no known exposure to rabies [23]

2 *Preparation and storage of sera* Rabies-hyperimmune horse serum is available commercially for the prophylaxis of rabies, and this serum is suitable for use in specificity tests for rabies virus For preparation of standard normal and rabies-hyperimmune serum it is preferable to use

a 20 per cent suspension in physiologic salt solution containing 2 per cent or more of inactivated normal guinea pig or rabbit serum

For titration prepare a series of 12 by 110 mm pyrex test tubes and label 10^{-1}, 10^{-2}, and so on in 10-fold steps through 10^{-6}. If the stock virus is a 20 per cent suspension, add 1.5 ml of the diluent to the tube labeled 10^{-1} and 2 7 ml. to the other tubes Take 1.5 ml. of the 20 per cent stock virus suspension and add this to the tube labeled 10^{-1} to make a 10 per cent suspension. Mix this by filling and emptying a 1 ml pipette 10 times, transfer 0 3 ml. to the tube labeled 10^{-2} and discard the pipette Use a 1 ml. pipette calibrated in 0 01 ml Mix with a clean pipette as for the prior tube, transfer 0 3 ml to the tube labeled 10^{-3}, and discard the pipette Subsequently, mix and transfer in this manner until the series is complete, using a clean pipette for each dilution

For inoculation of mice begin with the greatest dilution, that is, 10^{-6}, so it will be possible to test all dilutions with the same needle and syringe After completing one dilution empty the syringe into the tube containing this dilution, then fill and empty once in the next more concentrated dilution before filling and testing in mice Inoculate at least 4 and preferably 6 mice with each dilution by injecting 0.03 ml. intracerebrally as described previously The LD_{50} of the virus is determined by the *Reed-Muench method as described elsewhere in this book.*

H Preservation of Rabies Virus

Stock virus should be stored in pyrex test tubes. Seal the tubes, using a natural gas-oxygen torch or an acetylene torch, and store in a mechanical refrigerator at −12° C or colder, or in a carbon dioxide ice chest. For storage at 4° C it is best to keep brain and salivary gland specimens in undiluted glycerol in a pyrex test tube

I. Pathogens Other than Rabies Virus to Be Ruled Out

Mice inoculated intracerebrally with brain and salivary gland specimens may become infected with a variety of specific viruses and other microorganisms which produce encephalitis or meningitis and symptoms of tremulous activity, convulsions, and paralysis Among the viruses that may be encountered are those of pseudorabies or Aujeszky's disease (cow, pig, rat, and dog), lymphocytic choriomeningitis (man, rodent, and dog), equine encephalomyelitis (man, horse, and mule), St Louis encephalitis (man), encephalomyocarditis virus (rodent), vaccinia virus (man), ectromelia virus (mice), herpes simplex virus (man), poliomyelitis virus Type II (man), and mouse encephalomyelitis virus (mice). Among bacterial diseases, infection with the *Listerella monocytogenes* organism (man, cow, and pig) is commonly confused with a virus disease Toxoplasma parasites (man, dog, and

using a diluent of physiologic salt solution containing 2 per cent or more of inactivated normal guinea pig or rabbit serum It may be necessary to passage the virus 2 or 3 times in mice in order to obtain a satisfactory concentration of virus in the brain tissue. It is necessary to have a preliminary titration of the virus before attempting a serum-virus neutralization test The instructions for titration of the virus have been given previously Prepare sufficient stock virus at the time of the preliminary titration so that at least 5 pyrex glass ampules, each containing 2 ml of the 20 per cent suspension, can be glass-sealed and stored in the mechanical freezer or CO_2 ice chest.

4 *Stock virus for the serum-virus neutralization test* Prepare a stock 20 per cent tissue suspension of virus as described above and transfer to a 12 by 110 mm test tube and label S-10^{-1}, that is, stock virus If stock virus is available as a 20 per cent suspension stored frozen, thaw this material and transfer to the tube labeled S-10^{-1} Knowing the titer of the virus suspension prepare a series of tubes for 10-fold dilutions, including 1 dilution beyond that producing a 50 per cent mortality at the time of the preliminary titration, for example, S-10^{-2}, S-10^{-3}, S-10^{-4}, S-10^{-5}, and S-10^{-6} Add to each tube 2 7 ml of the diluent of physiologic salt solution containing 2 per cent serum Transfer 0.3 ml of the suspension from the tube labeled S-10^{-1} to the tube labeled S-10^{-2} and discard the pipette Set this tube apart from the others until it is mixed Mix this dilution with a clean pipette and transfer 0.3 ml to the tube labeled S-10^{-3} Continue in this way, using a clean pipette for each 10-fold dilution, until the series is complete

5 *Preparation of serum-virus mixtures* Set up one row of 12 by 75 mm test tubes for the normal serum control and label N-10^{-1}, N-10^{-2}, etc, through N-10^{-6} Set up another row of the same kind of tubes for the rabies-immune serum and label I-10^{-1}, I-10^{-2}, etc, through I-10^{-6} To each tube of the N- series add 0 3 ml of the normal control serum and using another clean pipette add 0.3 ml of the rabies-immune serum to each tube of I- series The pipettes used for measuring the serum and the virus dilutions added to the tubes should be graduated in 0 01 ml

Take 0 6 ml of the S-10^{-1} stock virus and measure 0 3 ml of this into the N-10^{-1} tube of normal serum and 0 3 ml into the I-10^{-1} tube of immune serum Discard this pipette and follow the same procedure with the remaining dilutions of virus, using a clean pipette for each dilution and adding the virus to the N- series tube first When the virus dilutions have been added to the serum tubes, mix by gently agitating the rack holding the test tubes, then place the rack in a 37 5° C water bath, with the water just covering the part of the tube containing the serum-virus mixture, or place the rack in an incubator set at 37 5° C and keep the tubes at this temperature for 1 hour At the end of this incubation period place the rack of tubes in the refrigerator for 1 hour The incubation per od is then complete, and the mixtures must be tested by inoculation into mice

6 *Testing the serum-virus mixtures* Test the contents of each tube in 6 mice, using a dose of 0 03 ml given by intracerebral inoculation Complete the immune serum series before testing the normal control

guinea pigs as a source of serum. Remove the food from the cages which hold the animals the night before they are to be bled so that a clear serum specimen may be obtained Take a good supply of normal serum before proceeding with immunization. The HEP Flury rabies virus at passage 180 or higher does not produce any disease in guinea pigs after intramuscular inoculation [19] Therefore, to immunize the guinea pigs give them each an intramuscular injection of 1.0 ml of a 10 per cent suspension of chick embryo tissue infected with HEP Flury virus Three weeks later give each of the animals an intramuscular injection of 1 0 ml of a 10 per cent suspension of guinea pig brain tissue infected with the Pasteur strain of rabies virus. Bleed the immunized animals 10 days after the 2d virus injection. Another injection of guinea pig brain tissue infected with the Pasteur strain of rabies virus, given after a rest period of 2 or 3 months, will produce a maximum antibody response. Prepare a stock suspension of the Pasteur strain of rabies virus from infected guinea pig brain and store this in the frozen state so that the same virus suspension will be available for booster immunization.

It is preferable to freeze-dry the normal and rabies-immune serum as soon as it is harvested and to store this material in sealed glass ampules under an atmosphere of nitrogen at a temperature of 4° C. For use rehydrate the desiccated serum with an equal volume of double distilled water It is convenient to use vaccine bottles with sleeve-type rubber stoppers for the storage of serum in the liquid state at 4° C. so that an aliquot of serum may be removed with a needle and syringe without opening the container Merthiolate (sodium ethyl mercury thiosalicylate) may be added as a preservative to a concentration of 1:10,000 for serum to be stored in the liquid state because this chemical substance has no significant viricidal activity at this dilution and does not appear to interfere with the serologic tests. However, if merthiolate is added to the rabies-immune serum, it must be added also to the control normal serum The virus-neutralizing activity of rabies-immune serum stored in the liquid state at 4° C ordinarily persists for several years Serum to be stored frozen should be inactivated by exposure to a temperature of 56° C. for 30 minutes before it is frozen. Complement and enzymes which are present in the serum become inactivated in a few days at 4° C or in a few weeks at —20° C The pH of the serum changes from 7.4 to 8 5 overnight when stored in a cotton-stoppered test tube as the CO_2 in the serum reaches an equilibrium with the air.

3. *Preliminary titration of the unknown virus* Prepare a 20 per cent suspension of mouse brain tissue infected with the unknown virus,

the rabies virus and therefore show specific neutralization by rabies-immune serum, whereas the mice inoculated with the more concentrated suspensions of the virus mixture die of the mouse disease

9 *Dilutions of serum versus constant amount of virus* This method of performing a serum-virus neutralization test is recommended for testing the potency of rabies immune serum Knowing the titer of the stock Pasteur strain rabies virus as determined by previous titration, dilute the virus suspension so that 0 03 ml will contain 200 LD_{50}, that is, double the concentration which is 2 log dilutions more concentrated than that producing a 50 per cent mortality in mice When this is mixed with an equal volume of serum or serum dilution, 0 03 ml will contain 100 LD_{50} of virus Table 3 shows the results of a cross neutralization test of a

TABLE 3

Cross-Neutralization Test, Vampire Bat Virus—Pasteur Rabies Virus

Serum	Serum Dilution Tested	Standard Virus	LD_{50} in 0 03 ml of Serum Virus Mixture 1 1 — Mouse Mortality* 100 LD_{50}	10 LD_{50}
	10^0		0/6	
Vampire bat virus-immune	10^{-1}	Vampire bat virus	0/6	
	10^{-2}		1/6	
	10^{-3}		4/6	
	10^0		0/6	
Vampire bat virus-immune	10^{-1}	Pasteur rabies virus	0/6	
	10^{-2}		0/6	
	10^{-3}		0/6	
Normal serum	10^0	Pasteur rabies virus	6/6	7/7
	10^0		0/6	
Pasteur rabies-immune	10^{-1}	Pasteur rabies virus	0/6	
	10^{-2}		0/6	
	10^{-3}		0/6	
	10^0		0/6	
Pasteur rabies-immune	10^{-1}	Vampire bat virus	1/6	
	10^{-2}		1/6	
	10^{-3}		6/6	
Normal serum	10^0	Vampire bat virus	6/6	6/6

* Results are shown in the form of fractions, with the numerator representing the number of mice that died 5 or more days after inoculation and the denominator representing the total number of animals used

serum series. One syringe and needle may be used for the immune serum series provided that the testing is done by starting with the maximum virus dilution and working backward to the most concentrated virus suspension. When changing dilutions, empty the syringe into the tube from which it was filled, then fill and empty once in the next serum-virus mixture before filling for test inoculation, working backward to the I-10^{-1} tube Another syringe and needle must be used for the normal serum-virus mixtures, which are tested last, begin with the maximum dilution of virus and continue in 10-fold increments to the N-10^{-1} tube.

7. *Observing inoculated mice and recording results* Hold the surviving inoculated mice for a period of 21 days before terminating the test and recording the mortality Record the results as shown in the sample protocol given below

Serum	Mortality Ratio of Mice Inoculated with Serum and Dilutions of Virus						LD_{50} Titer of Virus	Mouse LD_{50} of Virus Neutralized
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
Normal (N)	6/6	6/6	6/6	6/6	3/6	0/6	10^{-5}*	——
Rabies immune (I)	0/6	0/6	0/6	0/6	0/6		$<10^{-1}$	>10,000†

* Logarithmic calculation of titer by the Reed-Muench 50 per cent end point method
† Antilogarithm of 10^{-4}, the difference between 10^{-1} and 10^{-5}

8 *Interpretation of the serum-virus neutralization test* If the unknown virus is rabies, the difference in titer in normal versus rabies-immune serum will be at least two 10-fold dilutions, that is, more than 100 LD_{50} If the difference in titer is less than 2 0 log, it is almost certain that the unknown virus is not that of rabies Titration of a Pasteur strain of rabies virus in the normal and rabies-immune serum is an added check on the unknown virus and the quality of the rabies-immune serum

Rabies virus maintained in mice may be lost and replaced by another virus such as mouse encephalomyelitis virus Most varieties of mouse encephalomyelitis virus usually produce symptoms somewhat earlier than the brain-fixed rabies virus, that is, on the 2d or 3d day So-called "highly invasive" strains of mouse passage rabies virus which overwhelm the immunity produced by vaccination with rabies vaccine must be checked for the presence of mouse encephalomyelitis virus. A single subpassage through rabbits will remove the mouse disease The mouse disease virus is not apt to be present in high titer. Hence the higher dilutions of mouse brain containing both viruses may produce infection only with

virus injected by the same route.[19] Therefore, mice of this age may be immunized by a single intracerebral injection of HEP Flury rabies virus. The immunization of the mice is accomplished by intracerebral injection of 0 03 ml of a 10 per cent suspension of whole chick embryo infected with the HEP Flury rabies virus For passage in chick embryos, an injection of 0.25 ml of a 20 per cent suspension of whole chick embryo from a prior passage is given by the yolk sac to embryos 7 days of age, and these are incubated at 36 5° C. for 9 or 10 days before the embryos are harvested To perform a cross-protection test, immunize at least 24 mice, and 3 weeks later challenge half of these with the virus in question and the other half with the Pasteur strain of rabies virus Use a 10 per cent suspension of infected mouse brain as the challenge virus and give 0 03 ml by intracerebral injection A like number of control mice of the same age must be tested with the 2 viruses in parallel with the immunized mice Inoculate the immunized mice first Mice may be immunized against rabies by vaccination with killed-virus rabies vaccine according to the method developed for testing the potency of rabies vaccine [33]

L THE COMPLEMENT FIXATION TEST

The complement fixation test with mouse brain antigen, as described elsewhere in this book, can be applied to the identification of rabies virus Blood serum from guinea pigs vaccinated for the production of rabies-immune serum, as described previously, is suitable for use in the complement fixation test.

IV REFERENCES

1 Pa

2 Tr

3 Pa

4 Gr
cana Hist Natural, 5 35–42, 1944

5 Johnson, H N Derriengue Vampire Bat Rabies in Mexico *Am J Hyg*, 47 189–204, 1948

6 Venters, H D, Hoffert, W R, Scatterday, J E, and Hardy, A V. Rabies in Bats in Florida *Am J Pub Health*, 44 182–185, 1954

7

8

9

10
Meeting U S Livestock San Assn, 357–362 1954

street rabies virus of vampire bat origin, compared with a Pasteur strain of rabies virus when a fixed amount of virus and dilutions of serum are used[5]

It will be noted that both immune sera seemed to be more effective in neutralizing the Pasteur rabies virus than the street rabies virus This result is to be expected when comparing high mouse passage and low mouse passage varieties of rabies virus

The percentage dilution of the stock 20 per cent virus suspension to be used as the standard virus can be calculated by interpolating the logarithmic figure obtained from the titration of the virus For example, if the mouse LD_{50} of the virus is $10^{-5.5}$ by 0 03 ml when tested as a 10 per cent suspension, the same amount of a $10^{-3.5}$ dilution will contain 100 LD_{50}, a difference of 2 logs, and the $10^{-3.3}$ dilution of the 20 per cent tissue suspension will contain 200 LD_{50} per 0 03 ml

Set up 2 tubes for the normal serum control. Label one NS-10^0 for the undiluted serum and the other NS-10^{-1} for the 10-fold dilution of the normal serum Test the rabies-immune serum undiluted and in serial 10-fold dilutions through 10^{-3} and label these IS-10^0, IS-10^{-1}, IS-10^{-2}, and IS-10^{-3} Use 0 3 ml of serum or serum dilution for the test Prepare the 10-fold dilutions of serum by transferring 0 5 ml to 4 5 ml of physiologic salt solution For more accurate measurement of the antibody content of rabies-immune serum, test also half-log dilutions of serum. To do this prepare an alternate series of tubes labeled IS-$10^{-0.5}$, IS-$10^{-1.5}$, and IS-$10^{-2.5}$, each containing 2 16 ml of physiologic salt solution Take 1 5 ml of serum or serum dilution and add 0 5 ml to the next 10-fold dilution containing 4 5 ml diluent and 1 ml to the next half log tube containing 2 16 ml diluent. Do not mix the half-log dilutions until the 10-fold dilutions are complete Beginning with the highest dilution of the half-log series work backward and mix each dilution with the same pipette Measure 0 3 ml of the standard virus into each tube of serum or serum dilution beginning with the normal serum control Incubate the serum-virus mixtures as described previously and test by inoculation intracerebrally into mice, beginning with the immune serum series

As an additional virus control, test the standard virus diluted with an equal volume of physiologic salt solution containing 2 per cent serum and label this tube VC-100 or 100 LD_{50} Prepare two 10-fold dilutions of this virus suspension by transfer of 0.3 ml to 2 7 ml of the diluent to obtain the VC-10 and VC-1; that is, the 10 and 1 LD_{50} dilutions

K The Cross-Protection Test

The cross-protection test is the best method of determining the relationship of 2 viruses If immunization with 1 virus produces resistance to infection with another virus, they are classified as the same disease agent. Further classification depends on tests of pathogenicity for various experimental hosts and on the ability of the virus to invade the tissues of natural and experimental hosts

Mice inoculated intracerebrally at the age of 4 to 6 weeks with the HEP Flury rabies virus at passage 180 or higher do not sicken or die but are resistant to infection with the more pathogenic varieties of rabies

INFLUENZA

Keith E Jensen, Ph D

Assistant Professor of Epidemiology, School of Public Health, University of Michigan, Ann Arbor

11 Habel, K (Chairman) Report on the Second Session WHO Expert Committee on Rabies *Wld Health Org techn Rep Ser*, No 82, 1953
12 Hurst, E W, and Pawan, J L An Outbreak of Rabies in Trinidad without History of Bites, and with the Symptoms of Acute Ascending Myelitis *Lancet*, 2 622–628, 1931
13 Hurst, E W, and Pawan, J L A Further Account of the Trinidad Outbreak of Acute Rabic Myelitis Histology of the Experimental Disease *J. Path and Bact*, 35 301–321, 1932
14 de Verteuil, E, and Urich, F W The Study and Control of Paralytic Rabies Transmitted by Bats in Trinidad, British West Indies *Tr Roy Soc Trop Med and Hyg*, 29 317–354, 1936
15 Leach, C N, and Johnson, H N Human Rabies, with Special Reference to Virus Distribution and Titer *Am J Trop Med*, 20 335–340, 1940
16 Duffy, C E, Woolley, P V, Jr, and Nolting, W S Rabies, A Case Report with Notes on the Isolation of the Virus from Saliva *J Pediat*, 31 440–447, 1947
17 Johnson, H N and Sellers, T F Rabies In *Diagnostic Procedures for Virus and Rickettsial Diseases* 1st ed New York, American Public Health Association, 1948, pp 219–242
18 Koprowski, H Experimental Studies on Rabies Virus *Canad J Pub Health*, 40 60–67, 1949
19 Koprowski, H Biological Modification of Rabies Virus as a Result of Its Adaptation to Chicks and Developing Chick Embryos *Bull WHO*, 10 709–724, 1954
20 Kanazawa, K I Sur la culture in vitro du virus de la rage *Jap J Exper Med*, 14 519–522, 1936
21 Webster, L T, and Clow, A D Propagation of Rabies Virus in Tissue Culture and the Successful Use of Culture Virus as an Antirabic Vaccine. *Science*, 84 487–488, 1936
22 Schwab, M P, Fox, J P, Conwell, D P, and Robinson, T A Avianized Rabies Virus Vaccination in Man *Bull WHO* 10 823–835, 1954
23 Johnson, H N Experimental Studies on the Duration of Immunity in Dogs Vaccinated against Rabies *Bull WHO*, 10 725–729, 1954
24 Sellers, T F Preparation of Sellers' Stain In *Laboratory Techniques in Rabies WHO Monograph Ser*, No 23, 32–35, 1954
25 Mallory, F B *Pathological Technique* Philadelphia W B Saunders Company, 1938, pp 86–87, 195–196
26 Roe, M A, Lillie, R D, and Wilcox, A American Azures in the Preparation of Satisfactory Giemsa Stains for Malaria Parasites Pub Health Rep, 55 1272–1278, 1940
27 Johnson, H N Rabies Chapter 26 in *Oxford Medicine* New York, Oxford University Press, 1943, v 5, pp 599–630
28 Kraus, R, Gerlach, F, and Schweinburg, F *Lyssa bei Mensch und Tier* Berlin, Urban und Schwarzenberg, 1926 p 201
29 Koch, J, Lyssa In Kolle, W, Kraus R, und Uhlenhuth, P, *Handbuch der pathogenen Mikroorganismen*, 3d aufl Jena, G Fischer, v 8 547–694, 1930
30 Johnson, H N The Significance of the Negri Body in the Diagnosis and Epidemiology of Rabies *Illinois Med J*, 81 382–388, 1942.
31 Johnson, H N and Leach C N Comparative Susceptibility of Different Strains of [illegible] 10
32 [illegible] eau de chien d'un virus [illegible] *Ann Soc belge méd* [illegible]
33 Kaplan, M M Potency-test Requirements of United States National Institutes of Health (NIH) In *Laboratory Techniques in Rabies WHO Monograph Ser*, No 23, 1954, pp 117–124

greater concentrations of specific antibody in convalescent phase sera than in samples taken during the early stages of illness. Serologic methods are technically simpler and are therefore used routinely, but diagnosis is not obtained until after the patient is well Technics of virus isolation and identification are not difficult, however, and can be performed in the usual bacteriologic laboratory.

Many variations of serologic and isolation methods are used but, in the interest of clarity, only one dependable technic will be described in detail for each procedure

II DIAGNOSIS OF INFLUENZA BY ISOLATION OF THE VIRUS

A Collection of Specimens

For the isolation of influenza virus from the acutely ill patient, pharyngeal washings are used Optimally, the washings should be obtained during the first 3 days of illness and while the patient is still febrile, although virus has been recovered as long as 7 days after onset. The patient should gargle not once, but 2 to 3 times, with 10 to 15 ml of diluent (broth, skimmed milk, or distilled water) in a paper cup Some infective material may be brought from the trachea into the pharynx if the patient will cough after gargling. The washings should be transferred to a closed tube for transportation to the laboratory and tested as soon as possible, if a delay of a few hours is necessary, the fluid should be kept chilled at refrigerator temperatures When longer periods of storage are unavoidable, the washing should be quickly frozen and stored at temperatures near −70° C but not so high as −20° C since virus deteriorates rapidly at this temperature.[3] Washings have been preserved and shipped in glycerol, but this is not ideal Virus will remain active in throat washings in broth (to which penicillin and streptomycin have been added) which have been stored at 4° C for a considerable length of time

B Isolation of Virus by Amniotic Sac Inoculation

Since 1940[4] influenza virus has been known to grow well when inoculated into the amniotic sac of the embryonated egg so that this is now considered the most sensitive method for detection of virus in human throat washings. Although several methods of amniotic inoculation have been developed,[5-7] the window technic[8] with various modifications

I INTRODUCTION

INFLUENZA is an acute infection of the respiratory tract, with an incubation period of 1 to 2 days characterized by sudden onset, chills, fever, and general aches and pains[1] In comparison with the constitutional reaction, the respiratory symptoms and signs are commonly slight, at least in the early stages. There is often a moderate leukopenia The acute course is usually of 3 to 4 days' duration unless bacterial complications occur. It is difficult to differentiate clinically the single case of influenza from other acute respiratory illnesses in the early stages, but groups of cases can often be suspected from the differences in clinical manifestations Influenza is usually recognized only when epidemics occur. In military installations the occurrence of outbreaks of illness with this symptomatology in seasoned personnel as *well as in recruits is characteristic of epidemic influenza*

The agents causing influenza are viruses that exert their primary pathologic effect in damaging the epithelial cells of the respiratory tract. Virus can be *recovered from respiratory secretions* or washings with comparative ease during the acute phase of illness

Three distinct immunologic types of influenza virus have been identified Type A[2] has been related to the most extensive and severe outbreaks Type B[24] is also associatd with widespread epidemic disease although localized prevalences and sporadic infections appear to be more common than with Type A Type C[25a,25b] has been encountered primarily in sporadic cases or limited outbreaks of mild illness, but antibody studies demonstrate that the infection is widespread The possibility has been suggested that a virus isolated in Japan from pneumonitis of the newborn[26a] *may be a Type D influenza virus*[26b], *antibodies to it have* been demonstrated in the general population, but its classification is not yet fully established Serologic responses to Types A, B, and C are sharply type-specific with no evidence of cross reactions; this specificity must be considered in diagnostic work Moreover, marked variation has occurred with Type A strains, those first recognized in 1947 have been [illegible] Type B strains.

will usually exhibit their relationship if hyperimmune animal sera of acute and convalescent *human sera of known antibody titers are used*

Diagnostic procedures include isolation of the virus or detection of

closed immediately with cellophane tape. At least 6 eggs should be inoculated with each throat washing to increase the probability of success and should be incubated window side up at 35° C Care should be taken in handling to avoid contact of the fluids with the sealing cellophane tape.

4. *Harvesting of amniotic fluid* Positive results may be obtained as early as 48 hours after inoculation, but as a rule more fluids will yield virus after 3 days The cellophane is moistened with 70 per cent alcohol and stripped off The eggshell above the dropped chorioallantois is cut away with small scissors or may be picked off with forceps The allantoic fluid is aspirated or poured off and is usually not tested for hemagglutinins The amniotic sac is picked up with forceps, and the fluid contents aspirated with an 18-gauge needle fitted on a 5 ml. syringe or with a pipette and rubber nipple This often requires patience and ingenuity since the volume of fluid is usually small and may be pocketed below the embryo Sometimes no fluid is present, in which case the cavity should be washed out with 1 0 ml of buffered saline or broth

5. *Testing of fluids for hemagglutinating activity* [8 9] Since amniotic fluid is very viscous and frequently contains debris (especially after the 13th to 14th day of incubation), it often gives false positive agglutination patterns when used in low dilutions Therefore, the fluid should be diluted 10 times by mixing 0 2 ml with 1 8 ml saline Aliquots of 0 5 ml are distributed in 4 tubes (Kahn, 12 by 75 mm) with evenly rounded bottoms Saline suspensions of washed chicken erythrocytes (0 5 ml of 0 5 per cent by volume) are added to 2 of the tubes and to the other 2 tubes a similar volume of 1 0 per cent guinea pig red blood cells or human cells A saline control should be included The cells should be thoroughly dispersed in suspension by shaking One set of tubes containing chicken and guinea pig cells, respectively, is set at room temperature (22°C), the other in the refrigerator at 4°C , for 45 to 60 minutes In the saline control tubes the red cells will have settled down to a small, sharply outlined button and will flow when the rack is tilted If sufficient virus is present the agglutinated cells, as they settle, will cover the entire bottom in a uniform film, sometimes with characteristically serrated edges of quite typical appearance (Figs 1 and 2) With influenza virus of Types A or B the chicken or guinea pig cells at either temperature may show the positive reaction, whereas with influenza C virus only the chicken erythrocytes at 4° C will be positive Influenza A strains often agglutinate only guinea pig cells upon isolation,[10] but B strains usually agglutinate cells from both animals

is most widely used because it is the surest means of proper inoculation

1. *Preparation of eggs.* Fertile eggs that have been incubated for 10 days are usually employed. The eggs should be candled with embryo uppermost, and the area over the embryo marked. A rectangular area about 10 by 20 mm is lightly scored with a drill over the marked area and centered over the longitudinal dimension of the egg A small wedge-shaped sector is cut in the rectangle, around which the eggshell is carefully cut through to the eggshell membrane, care taken to avoid hemorrhage. This should be done shortly before inoculation, and the shell swabbed with 70 per cent alcohol The small triangular piece of shell is carefully removed first with a dissecting needle or fine forceps so as not to puncture the underlying membrane A hole is punched through the shell at the air sac end A drop of sterile saline may be placed on the exposed eggshell membrane to reduce trauma. A hole is teased in this membrane with a sharp needle, without puncturing the chorioallantoic membrane. By applying gentle suction with a rubber bulb over the air sac hole, the chorioallantoic membrane will drop with removal of air from the air sac, and with introduction of air through the hole at the operation site. After the membrane has dropped, the rest of the scored area is cut away with scissors or forceps

2. *Preparation of inoculum* The inoculum is prepared by mixing untreated throat washings with sufficient penicillin to bring the concentration to approximately 500 units per ml and streptomycin to provide a level of about 125 μg. per ml. Injections of this preparation may be made with tuberculin syringes fitted with half-inch 24- to 26-gauge needles.

3. *Inoculation into the amniotic sac* The amniotic sac surrounds the embryo and lies under the chorioallantoic membrane In order to gain entrance to this cavity, the amniotic membrane should be pulled out through a hole in the chorioallantois With small forceps a hole is forced through an avascular area of the chorioallantois and the elastic amniotic membrane picked up and pulled out into a long nipple-like protrusion, narrow at the base The needle is inserted at the base of the nipple, and 0 1 to 0.2 ml of washing is injected If some air from the syringe is also injected, the path of the bubbles will indicate whether the injection was into the proper cavity If by mistake the inoculation is made into the chorioallantoic cavity, the bubbles will not remain localized but will float off to the side. When the amniotic membrane is released it will return to its original site. After inoculation the window in the shell should be

titers are used, repeated injections may be necessary. Animals may be bled from the heart 10 to 21 days after injection Some difficulty may be encountered in obtaining satisfactory separation of serum from chicken blood. It is suggested that the clot be cut into small pieces and allowed to stand overnight in the icebox and then 5 to 6 hours at room temperature A high level of antibody is desirable, and the antiserum should titrate approximately 1 500 by the hemagglutination-inhibition pattern method The preimmunization sera should not be inhibitory at a dilution of 1 32 Higher titers of antibody may be obtained by hyperimmunizing animals with several injections The sera will give more strain-specific results, however, if the animals are bled 10 to 14 days after a single injection Sera may be stored in the fluid state with or without merthiolate, or dried from the frozen state in 1 ml amounts and kept at 4° C

C Other Methods for Detection of Influenza Virus in Throat Washings

Several other animals and tissues are susceptible to infection with influenza viruses and have been used successfully to demonstrate virus in throat washings Ferrets, hamsters,[12] and mice may be infected by intranasal inoculations Evidence for infection is obtained by serologic response or lung lesions and death, or subsequent inoculation of turbinate and lung suspensions into embryonated eggs

Isolations can also be achieved by the use of tissue culture technics, including minced chick embryo[13] or chorioallantoic membrane[14] and monkey kidney cultures[15]

Other technics have been advanced as screening devices or tools for more rapid diagnosis These include (a) attempts to detect hemagglutinins in throat washings,[16,17] (b) assays of normal inhibitory materials in saliva,[18] and (c) the use of cytologic staining methods to demonstrate viral antigen[19,20] None is yet in general use, however

III DIAGNOSIS BY SEROLOGY

A General Considerations

Serologic methods depend on the stimulation of antibody production during infection with influenza virus Since most persons have influenza antibodies resulting from previous antigenic experiences, it is necessary to compare concentrations of antibody in the acute and convalescent phase sera to determine whether there has been an increase in titer The 1st serum sample should be taken not later than the 5th day of disease and the 2d specimen at least 10 days after onset The rise in antibody titer may be measured on paired sera in several ways: by hemagglutination-inhibition and complement fixation tests or by neutralization in mice or embryonated eggs.

It is possible to reach a rapid presumptive diagnosis of the type of influenza virus with these primary agglutination tests as follows.

	4° C.		22° C.	
	Chick	Guinea Pig	Chick	Guinea Pig
A	±	+	±	+
B	+	+	+	+
C	+	—	—	—

6 *Treatment of fluids.* If fluids of the initial passage are negative or questionable, a further passage is indicated and justified. Pooled amniotic fluids should be inoculated into the amniotic sac of 10-day-old embryonated eggs. Antibiotics should again be added to the passage fluids. More than 2 or 3 negative passages are usually not profitable. Positive fluids should be checked by (*a*) subinoculation, using the allantoic or amniotic routes, and (*b*) serologic identification

(a) Subinoculation If Type C virus is suspected on the basis of cells agglutinated, the amniotic route is necessary for subinoculation; however, strains of Type A or B usually grow well in the allantoic cavity after primary passage The passage fluids are diluted 10^{-2} or 10^{-3} in the selected diluent with penicillin and streptomycin added.

(b) Serologic identification or typing of strains. Normal and immune phase sera prepared against strains of each antigenic type are diluted and tested with standard amounts of virus antigen as described in sections III B and III D Specific reactions with the antisera indicate the antigenic type of the isolate Occasionally isolates do not react well with antibody[11] and must be passaged several times in the laboratory *before typing can be achieved Suitable typing sera may be prepared*, using ferrets, hamsters, mice, rabbits, or chickens. Paired human sera with type-specific antibody rises against one antigenic type of influenza virus, and very low titers of activity against strains of other types can also be employed. Antisera may be prepared in animals susceptible to infection (ferret, hamster, and mouse) by intranasal inoculation of dilutions of infected allantoic fluid After two weeks, samples of blood are obtained and the sera are separated from the clots To immunize rabbits and chickens, allantoic fluids containing an approximate hemagglutinin titer of 1:320 or greater are desirable. After preliminary bleeding, the animals are injected with 2.5 ml. to 5.0 ml. intravenously and 5 to 10 ml intraperitoneally When fluids with lower hemagglutinin

the eggshell at the air sac end The hole is ground with a round burr fitted in a dental drill or utility tool or is punched with a blunt dissecting needle or diamond-point pencil Inoculum is injected with a 1-inch, 23-gauge needle, and the hole sealed with melted paraffin After incubation for at least 48 hours at 35° C allantoic fluids are harvested with pipettes or syringe and needle Fluids from several eggs are pooled and clarified by low speed centrifugation

b Titration of virus In the test to be described, a standard concentration of hemagglutinin is used to test different dilutions of serum Therefore, it is necessary to know the strength of the hemagglutinin in the virus suspension and to adjust by dilution to obtain the standard amount of activity to be added to the serum

Serial 2-fold dilutions are made by mixing 0 5 ml volumes of virus with 0 5 ml saline, using a separate pipette for the mixing and transfer of 0 5 ml of dilution to each subsequent tube and carrying the dilution series through 8 to 10 tubes * Then 0 5 ml of a 0 5 per cent red cell suspension is added, and the rack of tubes shaken to obtain an evenly dispersed suspension Racks are allowed to stand at room temperature (temperatures above 26° C should be avoided if possible) until the cells in the saline control tubes have settled Without disturbing the individual tubes, the results are read by examining the bottoms of the tubes in the respective series Individual tubes are usually graded as positive or negative Usually the end points are sharp, going from a diffuse film in one tube to a completely negative, sharply outlined button in the next (Fig 1) When partial agglutination does occur, it may be declared negative or may be used to interpolate the titer as being halfway between the 2 dilutions This calls for some judgment, and subjective standard should be constant The dilution of virus suspensions in the last positive tube is considered to be the titer, and this dilution therefore contains 1 agglutinating unit Titer is expressed as the ratio of allantoic fluid to diluent obtained in the last positive tube before the [illegible]

c Preparation of standardized antigen The suspensions of strains are diluted with saline so that each contains 4 hemagglutinating units per 0 25 ml Since the preliminary titrations are carried out with 0 5 ml volumes, it is necessary to adjust the concentration of virus to permit

* Tubes should be similar in size to the Kahn tube (13 by 75 mm) with an even hemispheric bottom The support should be a wire rack with mesh bottom to permit observation of the tests from underneath

B Serologic Methods Employing Red Cell Agglutination

The ability of influenza virus to agglutinate red cells furnishes a basis for measuring antibodies, since the virus when mixed with specific antibody is no longer capable of agglutinating erythrocytes The reaction is referred to as hemagglutination-inhibition This method for diagnosis gives results with human sera which correlate well with complement fixation and in vivo neutralization studies and is simpler to perform than the other tests There are two general ways of demonstrating agglutination and its inhibition, (*a*) the pattern test of Salk[21], and (*b*) the densitometric method described by Hirst and Pickels[22] The first will be described in greater detail as it is most suited for use in a general laboratory.

1. *The pattern method* There are numerous modifications requiring various types of containers, different volumes and concentrations of reagents, and so on The procedure to be described first in detail has been developed for routine use at the School of Public Health of the University of Michigan and represents, with few modifications, the method suggested by the Committee on Standard Serological Procedures in Influenza Studies[23]

a Reagents and materials.

(1) Erythrocytes The most satisfactory are red cells from an adult chicken, obtained from the heart or a wing vein, or by collecting at an abattoir The syringe or receptacle should contain about a 1/5 volume of 5 per cent sodium citrate to prevent clotting The cells should be washed at least 3 times in 2 to 3 volumes of saline to remove plasma elements The final centrifugation should be for 10 minutes at 1,500 r p m Cells are diluted to 10 per cent suspensions, by volume, and stored at 4° C They remain usable in this state from 5 to 7 days Blood may be stored for longer periods when mixed with an equal volume of Alsever's solution When it is not convenient to use avian cells, human erythrocytes may be substituted (Type O to avoid blood group reactions) and treated in the same manner except that longer centrifugation is necessary The concentration used in the test is 0 5 per cent (1 0 ml of the stock 10 per cent suspension with 19 0 of saline)

(2) Virus suspension Influenza viruses of Types A and B have most commonly caused epidemics Antibodies produced against strains of one type do not react with the other It is necessary, therefore, to include strains of each type for serologic diagnosis Almost every laboratory engaged in active research in influenza has a different set of prototype strains These usually consist of an older Type A strain such as PR8, isolated in 1934, and more recently isolated strains—FM1-1947, England-1951, or Malaya-1954—and Type B strains such as Lee (1940) and Allen (1945) or Great Lakes (1954) Type C strain may be included

Strains of virus are cultured in the allantoic sac of embryonated eggs that have been incubated at 37° C for 11 days Seed virus is diluted 10[illegible] to 10[illegible] in nutrient broth or saline, and 0 1 ml inoculated into the allantoic cavity through a hole in

heat labile materials in the serum, with no marked lowering of antibody levels Trypsin is not stable at this hydrogen ion concentration, and the usual practice is to prepare fresh solutions for each day's work The powdered trypsin preparation goes into the solution slowly, and first an opalescent suspension is obtained After repeated shaking at intervals of a few minutes, a crystal clear solution will be obtained, a stage important to reach, for a satisfactory digestion reagent.

Since diagnostic rises in antibody titer may be observed without treatment of sera in most cases, some prefer to remove nonspecific inhibitors only from those paired sera with which a rise in titer had not been observed in preliminary tests This is considered a labor-saving device when large numbers of sera are under test All sera are heated routinely at 56° C for 30 minutes, however, to destroy heat labile materials which may cause hemolysis of cells in the test

e Dilution of sera Acute and convalescent paired sera must be tested at the same time, and it is most convenient to test them against several strains of virus by making dilutions in volumes adequate for distribution to the required number of tubes Two-fold dilutions of serum, 0 25 ml per dilution, are made, using the same type of test tubes employed for the hemagglutinin titration The usual initial serum dilution is at least 1 8, although other dilutions are tested when retitrations appear warranted to demonstrate antibody rises If titrations were to include tests with 6 strains the operating procedure would be as follows:

(1) Treat 0.45 ml serum with 0 23 ml trypsin when inhibitor removal is necessary, otherwise only heat at 56° C. for 30 minutes. Then bring to a total volume of 3 6 ml with buffered saline to make an initial dilution of 1 8

(2) Set up 6 racks, each holding a series of 10 tubes.

(3) In 1 of the racks add 1 5 ml of saline to each of the last 9 tubes

(4) Distribute 25 ml of the 1 8 dilution of serum to the 1st tube in each series.

(5) Transfer 1 5 ml to the 1st dilution blank

(6) After thorough mixing, distribute the 2d dilution to the 2d tube in each series and transfer 1 5 ml *to the 3d tube in the dilution series.* This should leave 0 25 ml. in the working tube

(7) Continue the progression and discard the extra 1.5 ml. from the last dilution tube

(8) The serum remaining in the original tube should be the serum

the use of 4 units in a 0 25 ml volume. For example, if the titer of the virus suspension was 1.640 in 0 5 ml. volume (and by definition contains 1 unit at that dilution), then a 1:160 dilution would provide 4 units per 0.5 ml. A dilution of 1:80 would be the correct dilution to be made, however, so that 4 units are contained in 0.25 ml The necessary dilution to be made then can be quickly calculated by the formula:

$$\frac{\text{Reciprocal of titer of virus suspension}}{8} = \text{standardized antigen dilution}$$

Since the titer may vary with different lots of red cells and some mistake in dilution may have occurred, it is advisable to retitrate the test dilution to make certain the correct amount of hemagglutinin activity is added to the test This is performed by making serial 2-fold dilutions, mixing 0 5 ml of the prepared dilution in 5 tubes with 0.5 ml. saline blanks and adding 0 5 ml of the 0.5 per cent red cell suspension to be used on the day of the test After settling, the first 3 tubes of the series should show the positive pattern and the last 2 tubes should be negative indicating that there are 8 units per 0.5 ml. or 4 units per 0 25 ml. in the test suspension The virus concentration may need to be adjusted by dilution (if more than 3 tubes were positive), or by addition of more virus (if less than 3 tubes were positive)

d Treatment of sera. It is now clear that unless some paired sera are treated for destruction or removal of nonantibody inhibitory materials, the true antibody levels may be obscured and no rise in titer observed.[27] Here again a number of methods are available including digestion with trypsin[28] or *Vibrio cholerae* filtrates[11] or incubation with solutions of periodate.[29] Of these, the digestion with trypsin will be described in detail since this method appears quite efficient Commercial preparations of trypsin are readily obtained, and a standard amount of enzymatic activity can easily be added to each test. Details concerning the preparation and use of active filtrates from cultures of *Vibrio cholerae* are given in the *World Health Organization Technical Report Series* [30]

The use of trypsin to destroy nonspecific inhibitors in sera was first reported by Sampaio and Isaacs,[28] using a highly purified crystalline preparation. Subsequently, less purified commercial trypsin (Difco) has been demonstrated to be equally effective The current operating procedure is as follows. for each 1 ml. of serum, add 4 mg. of trypsin dissolved in 0 5 ml of 0.1 M phosphate buffer, pH 8 2 The trypsin-serum mixture is immediately incubated at 56° C for 30 minutes During this time the trypsin digests the inhibitors and is itself heat-inactivated, as are the other

strain constitutes the basis for diagnosis of influenza infection with a strain of the corresponding antigenic type

(5) Interpretations of diagnostic rises may also be influenced by recent influenzal vaccination experience If the patient has been vaccinated recently, the rise demonstrated with the 2 specimens may only indicate rising titer from vaccine stimulus This is especially true when an adjuvant vaccine has been given,[31] where antibody titer may continue to rise from 4 to 6 weeks after immunization In these cases complement fixation tests with "soluble antigen" may differentiate antibody stimulated during illness from the vaccinated response

(6) It is desirable to include standard human sera in each day's test with known titers of antibody for the test strains Although not absolutely necessary for diagnostic purposes, it does provide a reference point and permits comparisons of titers found in various sera on successive days When even greater precision of measurement is desired, each serum may be titrated in quadruplicate, or fractional dilutions of sera in steps of 0 1 log unit may be tested [32]

(7) When a large number of tests are to be performed, some mechanical device for delivering saline, virus suspensions, and red cells is of great assistance A mechanical pipetting machine either of the hand-operated syringe type (B-D Cornwall syringe) or a motor-driven type may be used

(8) Human O type cells may be substituted for avian cells, although their use makes slight changes in technic necessary The human cells settle more slowly, and one must wait longer (60 to 75 minutes) for the cells to sediment before reading the test

b Other pattern methods

Several useful modifications of this technic have been developed recently and will be described briefly

(1) Use of depression plate Dilutions are carried out as described in preceding sections and distributed to cups in plastic plates or made directly in these plates [33,34] The greatest value of this device lies in avoiding the necessity of individual glass test tubes and in ease of handling and cleaning the plates when the test has been completed

(2) Use of constant serum with different dilutions of virus [33 35 36] Some prefer to add a single dilution of serum (for example, 1 100 initial dilution) to different dilutions of virus The end point is then read in terms of the numbers of hemagglutinating units inhibited by the prepared dilution of serum This procedure provides a rapid screening device Titers obtained are somewhat different from those found with the inverse method

2 *The densitometric method* This method employs an indirect means of measuring agglutination in which the density of unagglutinated cells is measured with a photometer The transverse density of a column

control to which no virus will be added If agglutination occurs in this tube, it may be necessary to absorb agglutinins from the serum with erythrocytes.

Several sera can be diluted and distributed in each rack so that the same virus antigen can be added to the entire rack.

f The test. When all sera have been diluted and distributed, 0 25 ml of the adjusted virus suspension is added to each series, and the racks are shaken vigorously and allowed to stand at room temperature for 30 minutes (Serum control tubes should receive 0 25 ml. of saline instead of virus) The suspension of chicken erythrocytes (0 5 per cent) is then added in 0 5 ml volume to all tubes and then mixed by shaking the racks to ensure even dispersal of red cells The tests are incubated at room temperatures (20° to 26° C) (Antibody levels against strains of Type C influenza virus are determined in the same manner except hemagglutination tests are incubated at 4° to 6° C) When the cells in the control tubes have settled to a distinct button, it is time to read the test, usually in 45 to 60 minutes The tests are read in the same manner as the hemagglutinin titration, and end points are usually sharp (Fig 2) The titer of the serum is the final dilution of serum in the last negative tube

g General considerations

(1) The serologic end points are usually sharp and unequivocal, but with some strains the end points may be more confusing since all strains are not ideal for this technic This is especially true with many recently isolated strains that have not had repeated laboratory passage Again, some subjective standards for interpretation must be assumed and held constant

(2) The incubation period before reading of tests may be varied over a wide interval without marked alteration of the results If the tests stand too long, however, many of the patterns formed begin to d sappear so that for maximal clarity the tests should be read as soon as the cells have settled out in control tubes This is especially true during the summer months in high room temperatures Tests should then be incubated in refrigerated rooms or in water baths maintained at 17° to 20° C with ice cubes

(3) A diagnostic rise in antibody titer between acute and convalescent specimens should be 4-fold or greater in magnitude, to be certainly valid From general *considerations of the manner in which the test is read, it seems* doubtful that a 2-fold rise has very high validity in a diagnostic sense Titers encountered may vary from less than 1 32 to 16,384 (final serum dilutions)

[illegible] be expected That is

series of strains as suggested in a previous section Significant rise in titer for any

these and other preparations may be found in the *World Health Organization Technical Report Series*, by the Expert Committee on Influenza of that organization.[30]

The detailed procedure for the complement fixation test given below is the unaltered set of laboratory directions prepared by Dr. Edwin H. Lennette, at the Viral and Rickettsial Disease Laboratory, California State Department of Public Health, and is the standard technic used in that laboratory with a variety of viruses and rickettsiae

Precautions:

Never deliver the last 0 1 ml from a pipette, i e, do not deliver beyond the 0 9 mark

Never blow out the final drop

Never measure less than 0 1 ml. in making dilutions of reagents, unless a special pipette, such as the Kahn, is used

Use a clean pipette for each dilution of reagent unless the highest dilution is pipetted first, then work from that to the lower dilutions

1 *Reagents*

a 0 85 per cent saline containing 1 0 ml of 10 per cent $MgSO_4 \cdot 7H_2O$ per liter

b *Sensitized cell suspension*

(1) Cells—Defibrinated or citrated sheep red blood cells, washed in saline 7 minutes at 2,000 r p m for three successive times, packed for 15 minutes at 2,000 r p.m , and made to a 2 per cent suspension

(2) Hemolysin—2 units contained in 0.25 ml are used in the test

a Preparation of stock solution of 1 100

94 ml saline

4 ml 5 per cent phenol in saline

Mix and add

2 ml hemolysin preserved in 50 per cent glycerin (store in refrigerator 1–6° C.)

b Titration

0 5 ml of 1 100 hemolysin	+ 4 5 ml saline	= 1 1,000 dilution
1.0 ml of 1,1,000	+ 4 0 ml	= 1 5,000
1 0 ml of 1 1,000	+ 7.0 ml	= 1 8,000
1.0 ml of 1 1,000	+ 9 0 ml.	= 1 10,000
1 0 ml of 1 10,000	+ 0 5 ml	= 1 15,000
1.0 ml of 1 10,000	+ 1 0 ml	= 1 20,000
1 0 ml of 1 10,000	+ 1 5 ml	= 1 25,000
1 0 ml of 1 10,000	+ 2 0 ml	= 1:30,000

0 25 ml. hemolysin dilutions (1 5,000 to 1 30,000)

0 20 ml 1 30 complement (0 2 ml comp + 5 8 ml saline)

0 25 ml of 2 per cent sheep cells

0 40 ml saline

Cell control = 0 25 ml sheep cells + 0.85 ml. saline

of normal red cells in a test tube changes very little with time as the cells settle out. When influenza virus is mixed with the cells, however, clumps are formed which settle much more rapidly than individual cells; also there is a clearing of the red cell column, which is measured photometrically.

Advantages of this method over the pattern technic include increased accuracy of end point determination which may permit a serologic diagnosis to be made on the basis of a 2-fold rise in titer and more objective reading of the tests. A recently developed procedure,[32] however, based on the use of a series of fractional dilutions in steps of 0 1 log unit, permits a degree of precision with the pattern method comparable to that obtained with the photometric method Its main disadvantages are the need for certain special equipment (special test tubes and a photometer) and the increased care in making dilutions and so on, necessary to extract the full value of increased accuracy In view of its limited application to routine diagnosis, with full use only in certain research problems, the method will not be presented and the reader is referred to the descriptive report of Hirst and Pickels[22] for detailed information.

C. The Complement Fixation Test

Complement fixation has been widely used as a test for antibody in influenza with excellent results, though it is perhaps less widely used than hemagglutination-inhibition procedures The test may be employed with a number of variations of detail,[37] none of which departs widely from the methods used for complement fixation in other systems.

Allantoic fluid preparations of virus make excellent complement-fixing antigens, but some investigators prefer to use extracts of infected chorioallantoic membranes which contain so-called "soluble antigens" of influenza virus. They consider such preparations preferable because fewer strain-specific reactions are encountered than when allantoic fluid virus is employed. In addition the preparations may be of value in diagnosis of cases occurring shortly after vaccination. Current experience in this laboratory indicates most cases may be differentiated from vaccine responses on the basis of antibody rises as detected with soluble antigens at serum dilutions of 1:8 or greater. The method is not infallible, however, since a few vaccinated persons, without illness, produce antibody increases measured with soluble antigen. Other cases diagnosed by hemagglutination-inhibition methods do not have antibody responses detectable with soluble antigen at the 1.8 dilution of serum Details of

TITRATION OF ANTIGEN

Antigen Dilution	Immune Serum Dilution						Negative Serum	Antigen Control
	1 8	1 16	1.32	1 64	1 128	1 256	1 8	
1 2	4	4	4	4	0	0	0	0
1 4	4	4	4	4	1	0	0	0
1 8	4	4	4	4	2	0	0	0
1 16	4	4	4	4	2	0	0	0
1 32	4	4	4	2	0	0	0	0
1 64	1	0	0	0	0	0	0	0
Serum control	0	0	0	0	0	0	0	
Control antigen	0	0	0	0	0	0	0	0
Previous lot of specific antigen	4	4	4	4	0	0	0	0

Dilutions of both antigen and serum are made in master dilution tubes, from which 0 2 ml of each is transferred to the corresponding row of the titration tubes.

All the reagents, except the hemolytic system, are added, the mixtures are shaken and incubated overnight at 4°–6° C After warming the tubes for 10 minutes in a water bath at 37° C, the hemolytic system is added, and secondary incubation is performed at 37° C for 15–20 minutes, or until the control tubes show complete clearing The highest antigen dilution showing 3+ or 4+ fixation with the highest dilution of serum is generally regarded as 1 unit (For practical purposes, the unit is sometrmes greater than this For example, if a 1 32 dilution of antigen gives 3+ or 4+ fixation with a 1 256 dilution of antiserum, and a 1 64 antigen dilution gives 3+ fixation with a 1 128 serum dilution, the unit may be considered as 1 64)

e Sera —Sera are inactivated for 30 minutes in a water bath at 60° C Because nonspecific reactions may be encountered with more concentrated sera, the initial dilution used in the test is 1 8, and serial 2-fold dilutions are made with [illegible]

saline mixture is inactivated and used as the starting material

2 *The Test* (Refer to Table 1)

Explanation of 4-tube complement control—The tubes containing 0 20 ml, 0 15 ml, and 0 10 ml of the diluted complement should show complete hemolysis The tube containing 0 05 ml of the diluted complement should show no hemolysis If this tube shows hemolysis, an excess of complement was used in

Shake and incubate in water bath at 37° C for ½ hour and read.

The highest dilution of hemolysin which shows *complete hemolysis* represents 1 unit. Two units are used in the test proper.

Example. If the 1:20,000 dilution shows complete hemolysis, 1:25,000 partial hemolysis, then one unit would be 0.25 ml. of a 1:20,000 dilution. Therefore, two units would be contained in 0.25 ml. of a 1:10,000 dilution of hemolysin. The entire amount of hemolysin required for the test is diluted to the appropriate concentration at one time.

Sensitize only enough cells for use in the complement titrations. (Mix the remaining cells and hemolysin together the following morning prior to use in the test.)

Sensitize a quantity of cells sufficient for the complement titration by pouring diluted hemolysin into an equal volume of 2 per cent sheep cells and rapidly pouring back and forth several times. Allow to remain at room temperature 10 minutes before use.

c. Complement.—Two exact units contained in 0.2 ml. are used in the test. Fresh, or lyophilized guinea pig complement restored to the original volume, is titrated in the presence of antigen.

Titrate the 1:30 dilution of complement as follows. Use a Kahn pipette (0.2 ml. pipette calibrated in 0.001 ml.) to measure the complement. Wipe excess serum off outside of pipette and deliver required amount to bottom of each tube.

Titration of Complement

Reagent	Amount, in milliliters, of reagent to be added to tube								
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Complement (1:30)	0.13	0.12	0.11	0.10	0.09	0.08	0.07	0.06	0.05
Antigen (as diluted for test)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Saline	0.27	0.28	0.29	0.30	0.31	0.32	0.33	0.34	0.35

Shake and incubate in 37° C. water bath for ½ hour and add 0.5 ml. sensitized cells to all tubes.

Shake and incubate in 37° C. water bath for ½ hour and read. The tube containing the least amount of complement showing complete hemolysis represents 1 unit. Two *exact* units contained in 0.2 ml. are used in the tests.

Example. If 0.09 of 1:30 dilution of complement equals 1 exact unit, then 0.18 of 1:30 dilution of complement equals 2 exact units.

$\frac{30 \times 0.2}{0.18}$ equals 1:33 dilution of complement

d. Antigen.—2 units contained in 0.2 ml. are used in the test.

Antigen titration is conducted by testing serial 2-fold dilutions of antigen against serial 2-fold dilutions of immune serum to obtain the optimal dilution of antigen that gives fixation. This procedure, often referred to as a "box," "block," or "lattice" titration, is illustrated by the following schema.

the test If the tubes containing 0 10 ml., 0 15 ml., and 0 20 ml of diluted complement do not show complete hemolysis, insufficient complement was used During the final incubation period, controls are examined after 15 minutes If the 4-tube complement control shows the proper amount of hemolysis, the tests are removed from the water bath at this time If insufficiently cleared, the complement control tubes are examined at frequent intervals during the succeeding 15 minutes, and the test racks are removed from the water bath at the time when the desired amount of clearing in the complement control tubes is observed

3 *General Considerations*

Paired or multiple specimens of sera from a patient are tested routinely against strains of Types A and B viruses at one time and subsequently, if indicated, against Type C virus Currently an A-prime strain designated Spirup, isolated in 1948, is substituted for the Type A PR8 strain as lower titers of antibody are observed with this strain and so reduces the number of dilutions necessary to six or eight for each serum specimen The Lee strain (1940) will detect Type B antibody and the JJ strain (1950), the Type C antibody

Only a 4-fold or greater rise in titer from the acute to the convalescent stage is considered significant of infection In other cases a very high titer of antibody in both sera may be interpreted as a "presumptive positive," with the assumption that the bloods were taken too late in the course of the disease to demonstrate a rise

The difficulties encountered with complement fixation are few, and these are discussed briefly in the first chapter of this book and more fully elsewhere[38]

D Other Serologic Methods

1 *Neutralization test in mice* This is the classical method of measuring influenza antibodies and makes use of the fact that the addition of serum antibody to active virus reduces or abolishes its capacity to infect and kill mice (neutralization). The test has been used extensively in the past for diagnostic work and for antigenic comparisons[39,40] but will not be described in full detail here Serial 4-fold dilutions of heated serum (56° C for 30 minutes) are prepared, to which equal volumes of active virus are added The virus suspension is previously diluted so that the final mixture contains from 100 to 1,000 of 50 per cent lethal doses of virus per 0 05 ml The virus-serum mixtures are inoculated intranasally into Swiss mice under light ether anesthesia, using 03–05 ml per mouse and 4 to 6 mice per dilution The mice begin to die after 4 or 5 days and should be autopsied to confirm the presence of pulmonary lesions The survivors after 10 days are killed and autopsied for evidence of lung lesions Fifty per cent mortality or lesion end points may be calculated The technic is slow and costly and is not in general use as a routine method of serologic diagnosis

TABLE 1
Complement Fixation Test for Influenza

Tubes	Serum ml	Saline ml	Antigen ml	Nonspecific antigen† *ml*		Comp ml		Sensitized cells ml	
erum under test	0 20*	—	0 20	—	Shake and Add	0 20	*Overnight* incubation at 4–6°C followed by 10 min in 37°C water bath	0 50	
erum control	0 20 (1 8)	0 20	—	—		0 20		0 50	
Nonspecific antigen control†	0 20 (1 8)	—	—	0 20		0 20		0 50	
Reagent controls									
Complement control for antigen Tube No 1	—	0 35	0 20	—		0 05		0 50	15–30 minutes in 37°C water bath‡
Tube No 2	—	0 30	0 20	—		0 10		0 50	
Tube No 3	—	0 25	0 20	—		0 15		0 50	
Tube No 4	—	0.20	0 20	—		0 20		0 50	
(Tube No 4 also serves as specific antigen control)									
Hemolytic control	—	0 40	—	—		0 20		0 50	
Sheep cell control	—	0 60	—	—		—		0 50	
Nonspecific antigen control†	—	0.20	—	0.20		0.20		0.50	

* Series of tubes, each containing 0 2 ml of a serial dilution
† Normal allantoic fluid or membranes prepared and diluted the same as the specific influenza antigen
‡ Read test when complement control tubes 2, 3, and 4 show complete hemolysis (see text)

15 Mogabgab, W J, Green, I J, Dierkhising, O C, and Phillips, I A Isolation and Cytopathogenic Effect of Influenza B Viruses in Monkey Kidney Cultures *Proc Soc Exper Biol and Med*, 89 654–659, 1955

16 Soloviev, V D, Shubladze, A K, and Meltzer, N I Study of the Phenomenon of Erythrocytes Agglutination with the Virus of the Grippe, Part II *Zhurnal mikrobiol, epidemiol, i immunobiol*, 26 (10–11), 21–23, 1943

17 Kalter, S S A Rapid Method for Detection of Influenza Virus During Epidemics *Proc Soc Exper Biol and Med* 74 607–608, 1950

18 Fazekas de St Groth, S Nasal Mucus and Influenza Viruses II A New Test for the Presumptive Diagnosis of Influenza Infection *J Hyg*, 50 491–501, 1952

19 Liu, C Diagnosis and Pathogenesis of Influenza Infection in Ferrets by Means of Fluorescent Antibody *Fed Proc*, 13 502–503 1954

20 Panthier, R, Cateigne, G, and Hannoun, C, Nouvelle technique d'étude du virus grippal, premières applications practiques *Ann Inst Pasteur*, 75 338–350, 1948

21 Salk, J E A Simplified Procedure for Titrating Hemagglutinating Capacity of Influenza-Virus and the Corresponding Antibody *J Immunol* 49 87–98, 1944

22 Hirst, G K, and Pickels E G A Method for the Titration of Influenza Hemagglutinins and Influenza Antibodies with the Aid of a Photoelectric Densitometer *J Immunol* 45 273–283, 1942

23 Committee on Standard Serological Procedures in Influenza Studies An Agglutination Inhibition Test Proposed as a Standard of Reference in Influenza Diagnostic Studies *J Immunol* 65 347–353, 1950

24 Francis, T, Jr A New Type of Virus from Epidemic Influenza *Science*, 92 405–408, 1940

25a Minuse E, Quilligan J J, Jr and Francis, T, Jr Type C Influenza Virus I Studies of the Virus and Its Distribution *J Lab and Clin Med* 43 31–42 1954

25b Taylor, R M Studies on Survival of Influenza Virus Between Epidemics and Antigenic Variants of the Virus *Am J Pub Health* 39 171–178 1949

26a Kuroya M, Ishida, N and Shiratori T Newborn Virus Pneumonitis (Type Sendai) II Report The Isolation of a New Virus Possessing Hemagglutinin Activity *Yokohama Med Bull*, 4 217–233, 1953

26b Jensen K E, Minuse, E and Ackermann, W W Sterologic Evidence of American Experience with Newborn Pneumonitis Virus (Type Sendai) *J Immunol*, 75 71–77, 1955

27 Hilleman M R and Werner J H Influence of Non Specific Inhibitor on the Diagnostic Hemagglutination Inhibition Test for Influenza *J Immunol*, 71 110–117, 1953

28 Sampaio, A A de C, and Isaacs A The Action of Trypsin on Normal Serum Inhibitors of Influenza Virus Agglutination *Brit J Exper Path* 34 152–158, 1953

29 Burnet, F M and Lind P E Recombination of Influenza Viruses in the De Embryonated Egg I The Use of Periodate-Treated Sera for *in vitro* Characterization of Influenza Virus Strains *Australian J Exper Biol and M Sc*, 32 145–151, 1954

30 World Health Organization Expert Committee on Influenza *First Report, WHO Techn Rep Ser*, No 64, 1953

31 Salk, J E, Bailey M L, and Laurent, A M The Use of Adjuvants in Studies on Influenza Immunization II Increased Antibody Formation in Human Subjects with Influenza Virus Vaccine in a Water-in Oil Emulsion *Am J Hyg*, 55 439–456, 1952

32 Horsfall, F L, Jr, and Tamm I Fractional Dilution Procedure for Precise Titration of Hemagglutinating Viruses and Hemagglutination Inhibiting Antibodies *J Immunol*, 70 253–259, 1953

33 Salk, J E A Plastic Plate for Use in Tests Involving Virus Hemagglutination and Other Similar Reactions *Science*, 108 749, 1948

34 *Lépine, P Méthodes de laboratoire appliquées à l'étude du virus grippal Bull W Id Health Org*, 8 683–723, 1953

2 *In ovo neutralization tests.* Antibodies may be titrated by infectivity neutralization tests using embryonated eggs and the hemagglutinin test as a check for infection in each egg[41,42] Here again 4-fold dilutions of sera are made, virus suspension added, and the mixtures incubated at 37° C. for 30 minutes The virus suspension should be diluted to contain from 100 to 1,000 of 50 per cent infective doses. Four to 6 eggs are injected allantoically with 0 1 or 0 2 ml of each serum-virus mixture Titrations of virus suspensions must be included in the test to determine accurately the infectivity of the preparations. After incubation at 35° C. for 3 days, 0 5 ml of allantoic fluid is aspirated from each egg and tested for hemagglutinins The titer of the serum is calculated as the highest dilution which will neutralize 50 per cent of the infectivity (Reed and Muench[43]). As an in vivo method of titration, it has the advantage over the mouse test given above in requiring less time for completion and in permitting the use of nonmouse-adapted strains of virus

IV REFERENCES

1 Stuart-Harris, C H. *Influenza and Other Virus Infections of the Respiratory Tract* London, Edward Arnold and Co, and Baltimore The Williams and Wilkins Co, 1953

2 Smith, W, Andrewes, C H, and Laidlaw, P P A Virus Obtained from Influenza Patients *Lancet*, 2 66–68, 1933

3. Penttinen, K The Effect of Low Temperatures on Vaccines of Influenza Virus and on Purified Influenza Virus *J Immunol*, 64 165–171, 1950

4. Burnet, F M Influenza Virus Infections of the Chick Embryo by the Amniotic Route I General Character of the Infections *Australian J Exper Biol and M Sc*, 18 353–360, 1940

5. Beveridge, W I B, and Burnet, F M The Cultivation of Viruses and Rickettsiae in the Chick Embryo *Medical Research Council Special Report Series*, No 256, 1946

6 Hirst, G K Direct Isolation of Human Influenza Virus in Chick Embryos *J Immunol*, 45 293–302, 1942

7 Taylor, R M, and Chialvo, R J Simplified Technic for Inoculating into the Amniotic Sac of Chick Embryos *Proc Soc Exper Biol and Med*, 51 328–330, 1942

8 McClelland, L., and Hare, R The Adsorption of Influenza Virus by Red Cells and a New In Vitro Method of Measuring Antibodies for Influenza Virus *Canad J. Pub Health*, 32 530–538, 1941

9. ... Embryos In-
fluenza

10 ... Human Tech-

11 ... tection

12. ... 105–
108, 1942.

13. Francis, T, Jr, and Magill, T. P Cultivation of Human Influenza Virus in an Artificial Medium *Science*, 82 353–354, 1935

14. Weller, T H, and Enders, J F Production of Hemagglutinin by Mumps and Influenza A Viruses in Suspended Cell Tissue Cultures *Proc Soc Exper Biol and Med.*, 69 124–128, 1948

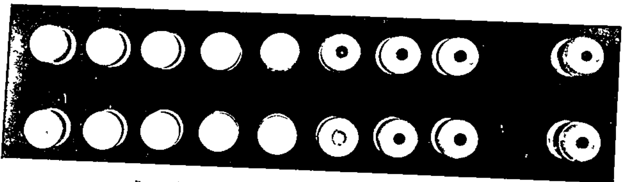


Fig 1 Photograph of bottom view of tubes, in a titration of hemagglutinins, showing patterns formed by sedimented erythrocytes in two different series of 2-fold serial dilutions of the same allantoic fluid containing Type A influenza virus Each tube contains 0 5 ml of virus dilution and 0 5 ml of 0 5 per cent chicken erythrocyte suspension The characteristic pattern of complete agglutination is seen in the first 5 tubes of each series A partial reaction is illustrated in the 6th tube in the lower row Readings are recorded as follows

	Dilution of Allantoic Fluid (before addition of cells)								
	80	160	320	640	1,280	2,560	5,120	10,240	End point
Top row	+	+	+	+	+	0	0	0	1,280
	60	120	240	480	960	1,920	3,840	7,680	End point
Bottom row	+	+	+	+	+	±	0	0	1,440

Taken from J E Salk 21

35 (Influenza, Diagnosis) Use of Red Blood Cell Agglutination Test in Study of Influenza, by Personnel of Naval Laboratory Research Unit No 1, United States Naval Reserve, University of California, Berkeley, Calif, *U.S Nav M. Bull*, 41:114–128, 1943
36 Sigel, M M, Shaffer, F W, Kirber, M W, Light, A B, and Henle, W Influenza A in a Vaccinated Population *J A M A*, 136 437–441, 1948.
37 Fulton, F, and Dumbell, K R The Serological Comparison of Strains of Influenza Virus *J Gen Microbiol*, 3 97–111, 1949
38 Lennette, E H An Evaluation of Diagnostic Procedures for Viral and Rickettsial Diseases Chapter 26 in *The Dynamics of Virus and Rickettsial Infections* New York, The Blakiston Co, 1954, pp 348–371
39 Magill, T P, and Francis, T, Jr Antigenic Differences in Strains of Epidemic Influenza Virus I Cross-neutralization Tests in Mice *Brit J Exper. Path*, 19 273–284, 1938
40 Smith, W, and Andrewes, C H Serological Races of Influenza Virus *Brit J Exper Path*, 19 293–314, 1938
41 Hirst, G K *In Vivo* Titrations of Influenza Virus and Neutralizing Antibodies in Chick Embryos *J Immunol*, 45 285–292, 1942
42 Burnet, F M, and Beveridge, W I B Titration of Antibody Against Influenza Viruses by Allantoic Inoculation of the Developing Chick Embryo *Australian J Exper Biol and M Sc*, 21 71–77, 1943
43 Reed, L J, and Muench, H A Simple Method of Estimating 50 Per Cent End-points *Am J Hyg*, 27 493–497, 1938

PRIMARY ATYPICAL PNEUMONIA

A. E FELLER, M.D.

Professor of Microbiology and of Medicine, School of Medicine, University of Virginia, Charlottesville, Virginia

and

MAURICE R. HILLEMAN, Ph.D.

Chief, Department of Respiratory Diseases, Walter Reed Army Institute of Research, Washington, D C

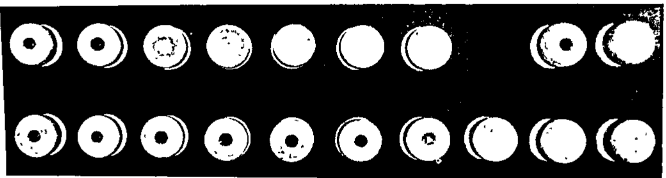


Fig 2 Photograph of bottom view of tubes in a hemagglutination-inhibition titration of an acute and a convalescent phase serum, showing typical patterns of sedimented erythrocytes Serum of the acute phase is in the top row and the convalescent serum in the bottom row The 1st tube in each series represents the negative reaction of the serum-cell control The 2 tubes in the upper right-hand corner of the photograph are a saline-cell control and a virus-cell control, respectively Readings are recorded as follows

		Final Serum Dilution									Inhibition Titer
	Control	32	64	128	256	512	1,024	2,048	4,096	8,192	
Acute	0	0	+	+	+	+	+	+	+	+	32
Conval	0	0	0	0	0	0	±	+	+	+	512

Taken from J E Salk 21

atypical pneumonia[6,7] These diseases may be bacterial, such as pulmonary tuberculosis, tularemia, or various bacterial pneumonias, they may be caused by viruses, such as the psittacosis-ornithosis group, influenza A, influenza B, or lymphocytic choriomeningitis, or they may be due to rickettsia, such as Q fever, and, finally, they may be due to fungi, such as coccidioidomycosis and histoplasmosis Occasionally, carcinoma of the lung may be confused with primary atypical pneumonia These diseases can usually be differentiated from primary atypical pneumonia by employing appropriate laboratory tests The diseases just listed, however, are usually responsible for a relatively small proportion of the patients presenting the signs, symptoms, and roentgenologic features of primary atypical pneumonia

Primary atypical pneumonia can usually be differentiated from the bacterial pneumonias on clinical grounds It is not sound practice, however, to make this differentiation in the absence of adequate bacteriologic studies of the sputum and blood[8] The absence of such features as shaking chill, pleuritic pain, rusty sputum, frank signs of pulmonary consolidation, and leukocytosis aids in distinguishing the disease from pneumococcal pneumonia In about 10 per cent of the cases the differential diagnosis from pneumococcal or other bacterial pneumonias may be difficult or impossible This is especially true early in the illness, when a decision is most desirable from the standpoint of therapy It should be emphasized that this difficulty in differential diagnosis may occur even though proper bacteriologic studies have been made

C Etiology

The problem of etiology in primary atypical pneumonia has occupied the attention of virologists for almost 2 decades During this period several different viral agents[9] were recovered in laboratory animals or embryonated eggs inoculated with specimens from patients with primary atypical pneumonia, but none has been generally accepted as causally related to human disease More recently, Hilleman *et al*[10] and Rowe *et al*,[11] employing human cell tissue cultures, reported their independent discoveries of a new family of viruses, certain of which may cause acute respiratory illness with frequent primary atypical pneumonia This group of viruses, which has been designated the RI (respiratory illness)[12] or APC (adenoidal-pharyngeal-conjunctival)[13] family and which includes viruses designated ARD (acute respiratory disease),[14,15] is antigenically heterogeneous, comprising a group of distinct serotypes * These serotypes may be distinguished readily in serum neutralization tests employing monotypic rabbit antisera[13,16] and in tests with the acute and convalescent serum specimens from selected patients with the infection[17] All the viruses of the family, however, elaborate a common group specific "soluble" complement-fixing (CF) antigen,[13,16-18] which is readily separable from the virus particles by filtration or by centrifugation procedures[18]

So far, 17 serotypes have been isolated, but insufficient data have accumulated either to assign each type to a particular clinical syndrome or to determine what proportion of the cases in a given clinical syndrome are due to the various serotypes Types 1, 2, 5, and 6 have been isolated almost exclusively from excised tonsils and adenoids and on only a few occasions from patients with respiratory disease Certain other types have been recovered only from cases of acute respiratory illness, as

* This group of agents has been recently designated adenoviruses[62]

I. INTRODUCTION

A. Definition

THE SYNDROME primary atypical pneumonia[1] may be defined as an acute infectious disease of the respiratory tract in which pulmonary infiltration is a prominent feature.[2-5] The pulmonary involvement is characteristically more evident in roentgenograms than by physical examination. The lesion tends to be patchy and less dense than that seen in pneumococcal pneumonia. Slight or moderate dullness to percussion and the presence of rales constitute the usual physical signs in the chest. The pulse and respiratory rates are either normal or only moderately increased. Constitutional symptoms (headache, malaise, chilliness, and fever) usually predominate over respiratory symptoms early in the illness Cough is invariably present later in the illness. Sputum is mucoid or mucopurulent The leukocyte count is normal or only moderately increased. As a rule, the illness is of mild or moderate severity and is similar in onset and symptomatology to the moderately severe acute respiratory diseases commonly termed nasopharyngitis, bronchitis, or "grippe" The febrile course is usually remittent, of 3 to 8 days' duration, and terminates by lysis. Occasionally, the disease may be severe, with extensive pulmonary infiltration, cyanosis, dyspnea, and rapid respiratory and pulse rates. Very mild or even asymptomatic cases have been recognized

B. Clinical Diagnosis

The diagnosis of primary atypical pneumonia depends to a large extent upon [illegible] is usually [illegible] nary [illegible] ex- [illegible] nical [illegible] picture which is exceedingly difficult or impossible to distinguish from primary

II PRIMARY ATYPICAL PNEUMONIA OF ADENOVIRUS ETIOLOGY

A Introduction

The laboratory diagnosis of infection with the adenoviruses may be accomplished either by the recovery and identification of the causative agent from the patient or by the demonstration of a significant (4-fold or greater) increase in amount of CF or neutralizing antibody in the patient's serum during convalescence from his illness Standardized technics for either of these tests have not yet been developed However, the procedures regularly employed in the Department of Respiratory Diseases, Walter Reed Army Institute of Research, are described below as tentative guides

B Laboratory Diagnosis

1 *Tissue cultures* * Tubes and bottle cultures of the HeLa strain of human epidermoid carcinoma cells are prepared by the general method of Scherer *et al* [33] At time of use, the bottle cultures (32 oz prescription size) should contain 10–20,000,000 cells and the tubes 60–90,000 cells Immediately prior to use and for the purpose of removing the human serum nutrient (which often contains RI virus antibodies), the contents of the bottles and tubes are washed 3 times with 20 or 1 0 ml, respectively, of 5 per cent chicken serum in Hank-Simms solution containing penicillin and streptomycin in a final concentration of 50 units of each per ml A mechanical device for simplifying the washing of tissue cultures in tubes has been described [34] Finally, 30 ml (bottles) or 0 6 ml (tubes) of cell maintenance solution are added A chicken serum maintenance solution (10 parts pooled chicken serum, 5 parts chick embryo extract ultrafiltrate, 85 parts Hanks' balanced salt solution plus antibiotics) has been most commonly used in the past and maintains the cells for 3 or 4 days A maintenance solution recently described by Ginsberg *et al* [35] provides more satisfactory preservation of cell viability for a longer period

2 *Virus recovery* The patient's throat washings are collected as early as possible after onset of illness (within 3 or 4 days) and stored

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* Tissue cultures and the necessary culture fluids are available commercially from Microbiological Associates, Incorporated, Bethesda, Maryland

described below, and additional types have been associated with conjunctivitis alone The whole problem of the relationship of the new RI viruses to the occurrence of the various respiratory diseases has been recently reviewed[19] and may be consulted for further details

Patients with respiratory illness caused by viruses of the RI family usually present fever with pharyngitis and cough Constitutional symptoms and, in addition, conjunctivitis, rhinitis, laryngitis, tracheobronchitis, and bronchiolitis may also be present.[20 23] Among epidemics in military recruit populations, around 15 per cent of the patients may develop an atypical pneumonia[20 21] The illnesses in the patients may be commonly classed as grippe, catarrhal fever, severe colds, acute pharyngitis, or virus pneumonia More specifically, they belong in certain of the respiratory disease categories described by the Commission on Acute Respiratory Diseases[24 26] during World War II, that is, undifferentiated acute respiratory disease (ARD), nonstreptococcal exudative pharyngitis, bronchitis resembling atypical pneumonia, and primary atypical pneumonia unassociated with the development of cold or streptococcus MG agglutinins Included also is the syndrome of pharyngoconjunctival fever recently described by Parrott *et al*[23] and by Bell *et al*.[22] Collectively, these entities belong in the more general categories of common respiratory disease[4] or of febrile catarrh described by Stuart-Harris *et al*[27] in 1938. The RI viruses do not appear to be responsible etiologically for the "common cold" in which there is a short course, a profuse and watery nasal discharge, and little or no fever.[14,15,21]

The primary atypical pneumonia cases in which cold or strepococcus MG agglutinins develop appear to be unassociated with RI virus infections Thus, patients *with proved RI disease fail to develop such agglutinins, and patients with the cold* or streptococcus MG agglutinin pneumonia do not present serologic evidence of RI virus infection[14,15,20,21,28] Primary atypical pneumonia associated with cold agglutinins also appears to be caused by a virus, based on the results of transmission studies employing human volunteers[29] The possibility that the bacterium streptococcus MG may play a role in the causation of the disease has not been entirely excluded but seems unlikely[30-32]

To summarize, primary atypical pneumonia is a clinical syndrome of diverse etiology. After exclusion of the clinically similar cases due to bacteria and other well-known causes, there is left the fairly distinct clinical syndrome described above under clinical diagnosis Recent data indicate that these cases can be divided into at least three goups (1) Adenovirus infections which clinically most often resemble ARD, but many of which have pulmonary infiltration and therefore may be recognized as primary atypical pneumonia So far, types 4 and 7 of the Adenoviruses have been demonstrated to be responsible for such cases (2) Cases of primary atypical pneumonia with cold hemagglutinins or streptococcus MG agglutinins, or both, in their sera This virus has not been isolated, but the disease has been transmitted to human volunteers employing bacteria-free filtrates of respiratory tract secretions (3) Cases not falling into either of the above groups As yet, insufficient data are available either to determine the proportion of total cases which may be placed in each of these three groups or to indicate whether or not further division of the groups will be possible as new data accumulate

virus[13,15,16 22,23] although other types may be involved In the tests, serial 4-fold dilutions of serum in the range from 1 2 to 1:512 are tested, and appropriate serum toxicity control tests (without virus) are always included Four-fold or greater increase in antibody titer is considered to be of diagnostic significance, in most cases the rise in titer is 8-fold or greater. While the primary neutralizing antibody response to infection is usually homotypic, this is not always true so that recovery and identification of the virus is necessary to determine definitively the infecting virus type

(2) Typing of virus strains Virus isolates are typed by the serum neutralization technic, using monotypic rabbit antisera[16] as described for human sera The rabbit antisera are usually highly strain specific, and the titer is usually 1 80 or greater

b Complement-fixation test

(1) Antigen Only a single viral antigen is necessary because of the group-specific activity of the complement-fixing substance To prepare antigen,* heavy cultures of HeLa cells in bottles are inoculated with 4 ml of undiluted seed stock (titer 10^{-1} or 10^{-2}) of the type 4 RI-67 strain, and the cultures are incubated at 36° C for 5 days Complete degeneration of the cells usually is accomplished by the 2d or 3d day, but higher CF titers are achieved by further incubation after this period The whole culture is homogenized in a Waring blendor, and the supernate obtained after centrifugation at 2,500 r p m for 20 minutes is the antigen The CF titer usually ranges from 1 8 to 1 32 As currently used, the virus in the antigen is not inactivated, and the CF activity of the preparation is stable when stored frozen at −20° C for at least 1 year Normal HeLa cell antigen for control purpose is prepared in identical manner from uninfected cultures

(2) CF technic For diagnosis, the patient's acute (4 days post onset or less) and convalescent (2 to 3 weeks) sera are titrated simultaneously for CF antibody The serum is inactivated at 56° C. for 30 minutes, and 0 25 ml amounts of serial 2-fold dilutions in the range from 1 5 to 1 320 are allowed to react with 2 exact units of guinea pig complement in 0 5 ml volume and 2 units of viral antigen in 0 25 ml amount or 0 25 ml of normal control antigen in the same dilution After incubation at 37° C for 1 hour, 0 5 ml of the hemolytic system which consists of a mixture of 0 25 ml of 3 0 per cent sheep cell suspension and 0 25 ml of physio-

* Now available from commercial sources

frozen at −70° C until tested For test, the thawed or freshly collected washings are centrifuged lightly to remove gross particles and then inoculated in 0.1 ml amount into 3 HeLa culture tubes. The cultures are incubated for 3 days in a stationary position and observed daily for the characteristic cytopathogenic change, which consists of rounding of the cells with darkening of the granular cytoplasm If no change occurs, the whole culture content is homogenized in a mortar or a Mickle tissue disintegrator* and the centrifuged supernate passed to 3 additional HeLa culture tubes Cultures showing specific degenerative change are passed to additional HeLa tube or bottle cultures to prepare seed virus or antigen stock for virus identification purposes. The virus may be identified as one of the adeno group by tests for the group-specific CF antigen in the culture, or it may be typed in serum neutralization tests with monotypic rabbit antisera prepared according to Rowe *et al.*[16] Virus recovery is usually possible in the throat washings of 50 to 70 per cent of patients giving diagnostic serologic tests

3 *Serologic procedures*

a Serum neutralization test For the neutralization test, one volume of diluted serum (inactivated at 56° C. for 30 minutes) is mixed with an equal volume of test virus suspension diluted to contain sufficient virus to cause complete degeneration of the cultures by the 3d day of incubation The virus seed lots usually titer 10^{-2}, and they can usually be employed in dilutions of 1:5 to 1 10 After incubation at 36° C. for 30 minutes, the serum-virus mixtures are inoculated in 0 1 ml amounts into 2 HeLa culture tubes which are incubated in a stationary position at 36° C and observed for 3 days. The neutralizing antibody titer of the serum is the highest initial dilution of serum that completely inhibits cellular degeneration by the virus

(1) Tests with patients' sera For diagnosis, the acute phase serum specimen, collected preferably within five days after onset of illness, and the convalescent serum are compared in neutralization tests run simultaneously The serologic response in infections in man is often homotypic only, necessitating the use of a "battery" of different viral types in the tests Types 3, 4, and 7 account[17,17a] for essentially all the adenovirus infections seen in military recruit populations at present, and these 3 types suffice for studies of military groups at this time. Most of the respiratory disease in civilian groups appears to be caused by type 3

* H Mickle, Hampton, Middlesex, England

apparently true of studies carried out in the military services. In one such study, cold hemagglutinins were found in only about 30 per cent of cases In civilian hospitals where the more severely ill patients would tend to be preferentially admitted, the occurrence of cold hemagglutinins may be as high as 75 to 90 per cent There is one report[40] that patients with primary atypical pneumonia treated with aureomycin developed lower titers of cold hemagglutinins and sustained them for a shorter time than the patients who were not given the antibiotic

The stage of the illness when the specimens of blood are taken, the temperature at which the blood is kept before the serum is removed, and the length of time and the temperature at which the sera have been stored may affect the results of tests Cold hemagglutinins are either absent, or are present only in low titer, during the first few days of illness, begin to appear either late in the 1st week or during the 2d week, are usually at their maximum in the period from the middle of the 2d week to the middle of the 4th week, and are either absent, or are present in considerably decreased titer, from the 4th to the 6th week Blood drawn for the purpose of testing for cold hemagglutinins should not be stored in the refrigerator before the serum is separated because the autologous cells will remove the cold hemagglutinins With serum of high titer, some of the agglutinins may be removed even at room temperature. The cold hemagglutinins, however, will elute from the cells if the blood is thoroughly warmed at 37° C before the serum is removed. Sera may be stored in the frozen state (−20° C) for at least three years without appreciable loss of titer of cold hemagglutinins[41] The titer of cold hemagglutinins may decrease in sera stored at 4° C for long periods, if stored at room temperature, the loss may be quite rapid[42-44]

The results of tests for cold hemagglutinins in a given serum may be considerably influenced by the density of the suspension of erythrocytes, by the age of the cells, and by the manner in which the tests are read When agglutination is read with the unaided eye, a 1 to 2 per cent suspension of cells will usually not show agglutination with "normal" sera in a titer higher than 8 to 16 With a 0.2 per cent suspension of cells the titers of some "normal" sera will range up to 32 or even 64 By using a light suspension of cells and reading the resultant agglutination by means of the microscope, titers in the range of 128 may occasionally be obtained in "normal" subjects Most workers have employed the density which appeared to facilitate the reading of lesser degrees of agglutination There is little agreement concerning the ideal density of erythrocyte suspension to be employed This is unsatisfactory because titers from different laboratories cannot be compared It seems desirable to adopt a density which will permit the highest degree of sensitivity compatible with ease of reading the tests It is suggested that a 0.2 per cent suspension fulfills these criteria[38] Erythrocytes older than 5 to 6 days should not be used since they are less

logic saline solution containing 3 units of sheep cell amboceptor are added, and the tests are read after final incubation at 37° C. for 30 minutes. The titer of the serum is considered to be the highest initial dilution of serum causing 3 + or greater fixation of complement

A 4-fold or greater rise in antibody titer of the patient's serum during convalescence is interpreted to indicate current infection with any of the known members of the adeno group. As in other diseases, the results of the serologic tests must be interpreted in the light of the clinical picture, since subclinical and inapparent infections with these agents are frequent[12,29] and, upon occasion, may occur concomitantly with another disease, for example, influenza.[20]

III. PRIMARY ATYPICAL PNEUMONIA ASSOCIATED WITH THE DEVELOPMENT OF COLD AND STREPTOCOCCUS MG AGGLUTININS

There are no specific laboratory tests as yet available to the diagnostic laboratory for the diagnosis of this disease There are two laboratory tests, however, which are useful adjuncts in differential diagnosis late in the course of illness They are the tests for cold agglutinins and agglutinins for streptococcus MG. Other serologic reactions have been described in primary atypical pneumonia, but since they are not of value in the diagnosis of the disease, they will be mentioned only briefly

A Cold Hemagglutinins

1. *General aspects.* The basis for this test is the observation that convalescent phase sera from many cases of primary atypical pneumonia contain agglutinins for human group O erythrocytes which are operative at temperatures ranging from 0° to 10° C With sera of high titer, agglutination may occur at 20° to 25° C. The homologous erythrocytes are also agglutinated in these temperature ranges At 37° C. the agglutination disappears.

The reported incidence of cold hemagglutinins in cases of primary atypical pneumonia has averaged about 50 per cent,[36,37] but the figures have varied widely. One factor which may account for this variation is the observation that cold hemagglutination can be correlated with the severity of illness as judged by the symptomatology, the extent of the pulmonary lesion, and the height and duration of the febrile response.[38,39] The lower figures reported may well have been the result of studies in which a high proportion of the milder illnesses were included. This was

as a check on the sensitivity and reliability of the tests from day to day Titers of the control sera should not vary more than ± 1 dilution from day to day.

3 *Interpretation of results* By employing the above technics, titers of 32 or 64 may be considered abnormal High titers—128 to 1,024 or more —are not often seen except in primary atypical pneumonia or in certain hemolytic anemias More important than the titer of a single serum is the demonstration that an increase in titer has occurred during the course of illness A 4-fold or greater increase in titer is probably significant Likewise, a definite decrease in titer late in convalescence is important To detect such changes in titer, it is important that the sera be collected at appropriate intervals (see above)

Cold hemagglutinins have been described in trypanosomiasis, blackwater fever, mumps orchitis, hemolytic anemias, certain diseases of the liver, peripheral vascular disease, and so on [47] Most of these diseases are not too difficult to differentiate from primary atypical pneumonia Cold hemagglutinins, usually in low titer, may also occur in a variety of the common respiratory diseases.[38 48 49 50] Experience has shown, however, that primary atypical pneumonia is the only respiratory disease which is likely to lead either to a large increase in titer in serial sera or to the presence of a high titer of cold hemagglutinins in a single serum specimen taken at the proper time after the onset of illness

It should be emphasized that the detection of cold hemagglutinins is not pathognomonic of primary atypical pneumonia and that failure to demonstrate the presence of the agglutinins does not exclude the diagnosis Since these agglutinins do not appear until late in the course of illness, the value of the test in differential diagnosis is usually retrospective

B. Agglutinins for Streptococcus MG*

1. *General aspects* Streptococcus MG [51] is a nonhemolytic streptococcus which appears to be distinct serologically from other species of streptococci The organism possesses a capsular structure which is responsible for its type-specific immunologic reactions It is immunologically related to, but antigenically distinct from, *Streptococcus salivarius*, type 1

Streptococcus MG is not easily distinguished from other varieties of

* Formerly called streptococcus 344

sensitive to agglutination than cells 2 to 4 days old[35,45] Apparently, fresh cells or cells 1 day old are also somewhat less sensitive than 2- to 4-day cells[45]

Erythrocytes from certain group O donors are more easily agglutinated than are those from other donors[45,46] There appears to be no way to determine which individuals possess cells that agglutinate readily except by comparative tests While these differences between donors are detectable, they are not of such a magnitude as to be of major importance With cells 2 to 4 days old, differences between the cells from different donors are minimized[45]

2 *Technic of test* The following technic[35] is recommended.

(a) Sera All sera from a patient should be tested on the same day to minimize day-to-day fluctuations in the tests. Sera need not be inactivated, but inactivation at 56° C for 30 minutes does not interfere with the test Sera should be as fresh as possible and must be taken at appropriate times after the onset of illness.

(b) Erythrocytes Defibrinated, oxalated, or citrated human group O blood may be employed The blood may be stored at 4° C. and will serve as a source of cells for 5 or 6 days[35,45] A suitable portion of the cells is washed 3 times with physiologic saline and then packed in the horizontal centrifuge at 1,500 r p m for 15 minutes. A 0.2 per cent suspension by volume of packed cells in physiologic saline is used in the test Unused parts of the 0 2 per cent suspension should be discarded each day.

Two-fold serial dilutions of the sera beginning with a 1 to 4 dilution are made in physiologic saline in 0 5 ml volumes Tubes measuring 12 by 100 mm are satisfactory. To each tube is added 0.5 ml of 0.2 per cent suspension of erythrocytes, the final dilution of serum in the first tube is 1 to 8 After thorough shaking, the racks of tubes are kept in the refrigerator at approximately 4° C overnight The following morning the tubes are removed from the refrigerator, one rack at a time, and read immediately before warming occurs. A fluorescent source of light and a black background are helpful Readings are graded from 4 +, which consists of a tight disc of cells that does not break up readily on gentle shaking of the tube, to 1 +, which is the least amount of definite clumping visible to the unaided eye. The end point is the highest final dilution of *serum in which definite (1 +) agglutination occurs* Positive tests must be read for the disappearance of agglutination after 30 minutes at 37° C. to eliminate heteroagglutinins or other antibodies which might cause agglutination Titers of cold hemagglutinins are expressed as the reciprocal of the final dilution of serum at the end point. If available, positive sera of previously determined titer should be included in each day's tests

be present in only about 20 per cent, in the more severe or prolonged illnesses they may be found in approximately 75 per cent of cases. Immunologic studies have indicated that cold hemagglutinins and agglutinins for streptococcus MG are not related [53] The observation that a positive test for streptococcus MG agglutinins is more likely to be found in a patient who has developed cold hemagglutinins may be explicable, at least in part, by the fact that each occurs more abundantly in severe cases

2 *Technic of test*

(a) Streptococcus MG Streptococcus MG may be obtained from the American Type Culture Collection where it is designated Streptococcus spp 9895 Isolation of the organism may be accomplished by employing the technic devised by Thomas, Mirick, Curnen, Ziegler, and Horsfall,[53] but the technic is difficult unless antistreptococcus MG serum is available

For continued subculture of streptococcus MG in the laboratory, beef infusion broth or Todd-Hewitt broth[53] may be employed When cultures are stored at 4° C for more than a week, defibrinated rabbit blood should be added to the broth in a concentration of 2 per cent Blood broth cultures remain viable for as long as 2 months at 4° C

(b) Materials and methods for agglutination test. The technic[53] is as follows

Bacterial suspensions for agglutination tests are prepared from 18-hour broth cultures of streptococcus MG The bacterial cells are washed 3 times in 0 85 per cent NaCl and suspended in sufficient physiologic saline to give a turbidity approximating No 5 in the McFarland scale. The streptococci are killed by heating at 65° C for 1 hour. Merthiolate, in a final concentration of 1 10,000, is added as a preservative

Serial 2-fold dilutions of unheated serum are made in physiologic saline in 0 5 ml volumes Each serum dilution is then mixed with an equal volume of streptococcal suspension The final dilutions of serum should range from 1 10 to 1 320 Dilutions lower than 1·10 are not used in routine tests because of the frequent occurrence of nonspecific agglutination of streptococcus MG in 1:2 or 1 4 dilutions of either normal human or rabbit serum Sera are not heated at 56° C, since the agglutination titer of convalescent serum may be reduced by heating at this temperature The tubes containing the serum and the streptococcal suspension are placed in a water bath at 37° C for 2 hours, followed by 18 hours in the icebox at 4° C. They are then again placed in the water

indifferent streptococci which inhabit the respiratory tract, notably *Streptococcus salivarius*, type 1, and *Streptococcus mitis*. However, it has been found[51] that streptococcus MG produces small fluorescent colonies on sucrose agar whereas *Streptococcus salivarius*, type 1, produces large succulent colonies. Various strains of *Streptococcus mitis* will ferment raffinose and produce marked greening on blood agar. These properties are not possessed by streptococcus MG, although definite but small degrees of green hemolysis may occur.

Streptococcus MG was first isolated from the lungs of fatal cases of primary atypical pneumonia[52] It has since been isolated from the upper respiratory tracts of normal persons and of persons with nonpneumonic acute respiratory disease[50,53] The organism, however, has been isolated from the respiratory tract secretions and lungs of cases of primary atypical pneumonia more frequently than from the other forms of acute respiratory disease[31] The part which streptococcus MG plays in the mechanism of primary atypical pneumonia is unknown. In the experiments in which primary atypical pneumonia was transmitted to human volunteers, streptococcus MG was isolated with almost identical frequency in the total group of volunteers before as well as after inoculation.[50]

More pertinent to the present discussion is the observation that many patients convalescent from primary atypical pneumonia develop antibodies in their blood for streptococcus MG. These antibodies may be demonstrated by agglutination of either the encapsulated organisms or nonencapsulated R variants, by precipitation with the capsular substance, by capsular swelling, or by skin reactions on intradermal injection of the capsular substance[53] Only the agglutinins will be considered here because the technic for their detection appears to be the most practical for use in most laboratories and is the only one which has received sufficient usage to permit of its evaluation[36,53-57] Although these agglutinins do not appear in all cases of primary atypical pneumonia and may be found in certain other conditions, their detection in convalescent phase sera is of value in the differential diagnosis of primary atypical pneumonia Since the antibodies do not appear until the 2d or 3d week after the onset of illness and reach maximum levels during the 4th and 5th weeks, the demonstration either that they are present in high titer or that a significant increase in titer has occurred is of value only in making a retrospective diagnosis As in the case of cold hemagglutinins, antibodies for streptococcus MG can be correlated with the severity of illness. In mild cases of primary atypical pneumonia the agglutinins may

4 The absence of the agglutinins does not exclude primary atypical pneumonia

5 Since the agglutinins do not usually appear until late in convalescence, the results of the tests are of value principally in the retrospective differential diagnosis of primary atypical pneumonia

C Other Serologic Reactions

The capacity of convalescent phase sera of cases of primary atypical pneumonia to fix complement with a variety of antigens, particularly those consisting of fresh tissue suspensions, has been demonstrated [61] The substances responsible for these reactions appear to be separable from those causing cold hemagglutination and agglutination of streptococcus MG [53] False positive serologic tests for syphilis have also been noted in primary atypical pneumonia [57] Such serologic reactions have not been demonstrated to be of practical value in the diagnosis of primary atypical pneumonia

IV REFERENCES

1 Primary Atypical Pneumonia Etiology Unknown Official Statement, *War Med*, 2 330–333, 1942

2 Dingle, J H, Abernethy, T J, Badger, G F, Buddingh, G J, Feller, A E, Langmuir, A D, Ruegsegger, J M, and Wood, W B, Jr Primary Atypical Pneumonia, Etiology Unknown *Am J Hyg*, 39 67–128, 197–268, 269–336, 1944

3 Dingle, J H Atypical Pneumonia *Advances Pediat*, 2 194–237, 1947

4 Feller, A. E Common Respiratory Disease Chapter 190 in Harrison, T R, *Principles of Internal Medicine* Philadelphia, The Blakiston Co, 1954, 2d ed, pp 1062–1079

5 J[illegible], J H Clinical Vari- [illegible] 20, 1951

6 D[illegible] monias II Primary [illegible] *Med*, 227 378–385,

7 Francis, T Jr Virus Pneumonia *Canad J Pub Health*, 35 49–54, 1944

8 American Public Health Association *Diagnostic Procedures and Reagents* 3d ed New York, 1950

9 Horsfall, F L, Jr Primary Atypical Pneumonia Chapter 17 in Rivers, T M, *Viral and Rickettsial Infections of Man* J B Lippincott Co, 1952, 2d ed, pp 383–391

10 H[illegible]

11 R[illegible]

12 H[illegible] iations *Am J Hyg*, 62 29–43, 1955

13 Huebner, R. J, Rowe, W P, Ward, T G, Parrott, R H, and Bell, J A Adenoidal-Pharyngeal Conjunctival Agents A Newly Recognized Group of Common Viruses of the Respiratory System *New England J Med*, 251 1077–1086, 1954

bath at 37° C for 2 hours, after which the tubes are shaken and readings of the degree of agglutination are made The final period of 2 hours at 37° C. is of importance since nonspecific agglutination of streptococcus MG may occasionally occur at icebox temperatures Such agglutination disappears when the mixtures are brought to 37° C.

In estimating the degree of agglutination, the following standards are employed. A designation of 4 + is assigned to tubes in which agglutination is complete, with a solid plaque or disc of bacteria and a clear supernatant fluid Agglutination with large clumps and clear supernatant fluid, but without complete settling of the bacteria to the base of the tube, is designated as 3 + Agglutination with incomplete clearing of the supernatant fluid is designated as 2 + Agglutination with turbid fluid, but with particles visible to the unaided eye, is designated as 1 + Agglutination which requires the use of a hand lens for visualization is designated as ± The agglutination titer is taken as the highest dilution of serum in which reactions of 1 + or more are observed.

3. Interpretation of results Titers of 1.20 or more may be found in approximately 50 per cent of cases of primary atypical pneumonia [31] However, the reported incidence has varied widely,[30,36] and in one series of cases was less than 10 per cent [59] As noted above, the agglutinins appear to occur more commonly in the more severe cases The agglutinins may be found in normal persons, in various acute respiratory infections, in certain streptococcal infections, and in a peculiar pneumonia associated with retinal cytoid bodies [55 56,60] They are usually present in low titer in these conditions.[31,36]

As in the case of cold hemagglutinins, the demonstration of an increase in titer of a 4-fold increment or more during convalescence is more important than the height of the titer in a single serum specimen It has been pointed out that a 4-fold or greater increase in titer of streptococcus MG agglutinins has almost never been encountered except in primary atypical pneumonia [31,36]

Several features concerning the interpretation of the results of tests for agglutinins to streptococcus MG must be kept in mind:

1. Sera must be obtained at the proper stage or stages of illness.

2. Low titers (1:10 or 1:20) must be interpreted with caution.

3. While significant increases in titer have been found predominantly, or solely, in primary atypical pneumonia, experience with the test has been limited

34 Hilleman, M. R , and Taylor, R O Mechanical Apparatus for Washing Tissue Cultures in Tubes *J Immunol*, 74·498–500, 1955

35 Ginsberg, H S , Gold, E , and Jordan, W S , Jr Tryptose Phosphate Broth as Supplementary Factor for Maintenance of HeLa Cell Tissue Cultures *Proc Soc Exper Biol and Med*, 89 66–71, 1955

36 Horsfall, F L , Jr Primary Atypical Pneumonia *Ann Int Med*, 27 275–281, 1947

37 Finland, M , and Barnes, M W Cold Agglutinins VII Tests for Cold Isohemagglutinins in Pneumonia and Other Acute Respiratory Infections over a Four Year Period *Am J M Sc*, 221 152–157, 1951

38 Commission on Acute Respiratory Diseases Cold Hemagglutinins in Primary Atypical Pneumonia and Other Respiratory Infections *Am J M Sc*, 208 742–750, 1944

39 Finland M , Peterson, O L , Allen, H E , Samper, B A , and Barnes, M W. Cold Agglutinins II Cold Isohemagglutinins in Primary Atypical Pneumonia of Unknown Etiology with a Note on the Occurrence of Hemolytic Anemia in These Cases *J Clin Invest*, 24 458–473, 1945

40 Gallagher, C D , and Gallagher, J R Effect of Aureomycin on the Development of Cold Agglutinins in Primary Atypical Pneumonia *New England J Med*, 247 314–316, 1952

41 Feller, A E Cold Hemagglutinins Effect of Storage at –20° C *J Lab and Clin Med*, 39 440–442, 1952.

42 Peterson, O L , Ham T H , and Finland, M Cold Agglutinins (Autohemagglu

43)

44

45 l

46 Young, L E The Clinical Significance of Cold Hemagglutinins *Am J M Sc*, 211 23–39, 1946

47 Stats, D , and Wasserman, L R. Cold Hemagglutination —An Interpretive Review *Medicine*, 22 363–424, 1943

48 Finland, M , Peterson, O L , Allen, H E , Samper, B A , and Barnes, M W Cold Agglutinins I Occurrence of Cold Isohemagglutinins in Various Conditions *J Clin Invest*, 24 451–457, 1945

49 Favour, C B Autohemagglutinins—"Cold Agglutinins" *J Clin Invest*, 23 891–897, 1944

50 ical kins

51 Ion- I stics to *Streptococcus salivarius*, Type I *J Exper Med*, 80 391–440, 1944

52 Thomas, L , Mirick, G S Curnen, E C , Ziegler, J E , and Horsfall, F L , Jr Serological Reactions with an Indifferent Streptococcus in Primary Atypical Pneumonia *Science*, 98 566–568, 1943

53 Thomas, L , Mirick, G S , Curnen, E C , Ziegler, J E , and Horsfall, F L , Jr Studies on Primary Atypical Pneumonia II Observations Concerning the Relationship of a Non-Hemolytic Streptococcus to the Disease *J Clin. Invest*, 24 227–240, 1945

54 Meiklejohn, G , and Hanford, V L Agglutination Tests with Streptococcus No 344 in Primary Atypical Pneumonia *Proc Soc Exper Biol. and Med*, 57 356–358, 1944

14 Ginsberg, H S, Badger, G F, Dingle, J H, Jordan, W S, Jr., and Katz, S Etiologic Relationship of the RI-67 Agent to "Acute Respiratory Disease (ARD)" *J. Clin Invest*, 34 820–831, 1955
15 Ginsberg, H S, Gold, E, Jordan, W S, Jr, Katz, S, Badger, G F, and Dingle, J H Relation of the New Respiratory Agents to Acute Respiratory Disease *Am J Pub Health*, 45 915–922, 1955
16 Rowe, W P., Huebner, R J, Hartley, J. W, Ward, T G, and Parrott, R H Studies of the Adenoidal-Pharyngeal-Conjunctival (APC) Group of Viruses *Am J Hyg*, 61 197–218, 1955
17. Hilleman, M R, Werner, J H, and Stewart, M T The Grouping and Occurrence of RI (Prototype RI-67) Viruses *Proc Soc Exper Biol and Med*, 90 555–562, 1956
17a Berge, T O, England, B, Mauris, C, Shuey, H E, and Lennette, E H Etiology of Acute Respiratory Disease Among Service Personnel at Fort Ord, California *Am J Hyg*, 62 283–294, 1955
18 Hilleman, M R, Tousimis, A J., and Werner, J H Biophysical Characterization of ... 1955
19. ... Respiratory
20 ... ak of Acute ... nard Wood,
21 ... in Patients
22 ... Pharyngo- ... zed Disease
23 ... ough, N M. ... ype 3 Ade- ... 251 1087–1090, 1954
24 Commission on Acute Respiratory Diseases Acute Respiratory Disease Among New Recruits *Am J Pub Health*, 36 439–450, 1946.
25 Commission on Acute Respiratory Diseases Clinical Patterns of Undifferentiated and Other Acute Respiratory Diseases in Army Recruits *Medicine*, 26 441–464, 1947
26 Commission on Acute Respiratory Diseases Epidemiology of Atypical Pneumonia and Acute Respiratory Disease at Fort Bragg, North Carolina *Am J Pub Health*, 34 335–346, 1944
27 Stuart-Harris, C H, Andrewes, C H, and Smith, W A Study of Epidemic Influenza

MUMPS

JOHN F ENDERS, Ph D.

Professor of Bacteriology and Immunology at the Children's Hospital,
Harvard Medical School, and
Chief of the Research Division of Infectious Diseases,
The Children's Medical Center,
Boston, Massachusetts

and

KARL HABEL, M.D.

Chief of the Laboratory of Infectious Diseases,
National Microbiological Institute,
National Institutes of Health,
U S. Public Health Service,
Bethesda, Maryland

55 Eaton, M D Serological Differentiation of Primary Atypical Pneumonia from Virus Pneumonia of the Psittacosis Group *Proc Soc Exper Biol and Med* 60 231–235, 1945
56 Finland, M, Samper, B A, and Barnes, M W Cold Agglutinins VI Agglutinins for an Indifferent Streptococcus in Primary Atypical Pneumonia and in Other Conditions and Their Relation to Cold Isohemagglutinins *J Clin Invest*, 24 497–502, 1945
57 Florman, A L, and Weiss, A B Serologic Reactions in Primary Atypical Pneumonia *J Lab and Clin Med*, 30 902–910, 1945
58 Todd, E W, and Hewitt L F A New Culture Medium for the Production of Antigenic Streptococcal Haemolysin *J Path and Bact*, 35 973–974, 1932
59 Commission on Acute Respiratory Diseases Unpublished data
60 Tumulty, P A, Berthrong, M, and Harvey, A M Peculiar Pneumonia Associated with Retinal Cytoid Bodies *Bull Johns Hopkins Hosp*, 88 239–263, 1951
61 Thomas, L, Curnen, E C, Mirick, G S, Ziegler, J E, and Horsfall, F L, Jr Complement Fixation with Dissimilar Antigens in Primary Atypical Pneumonia *Proc Soc Exper Biol and Med*, 52 121–125, 1943
62 Enders J F *et al* Adenoviruses Group Name Proposed for New Respiratory Tract Viruses *Science*, 124 119–120, 1956

I. *CLINICAL FEATURES OF MUMPS*

For reviews of the clinical and epidemiologic aspects of mumps, see References 1, 2, 3, 4, and 5.

A Mode of Transmission

Mumps is transmitted from one individual to another by saliva containing the virus. Transmission may be through direct transfer, air-suspended droplets, or fomites recently contaminated with saliva.

B Pathogenesis

Although the pathogenesis of mumps infection is not yet precisely understood, the evidence available[5] strongly supports the following conception Virus first multiplies in an unknown site, presumably in the upper respiratory tract. Invasion of the blood stream then occurs and infection of the salivary glands and other organs is thus established It is probable, however, that secondary involvement of other organs is often accomplished by the extension of virus from previously infected salivary glands

2 Procedures for serial passages
    a Amniotic sac inoculation
    b Yolk sac inoculation
    c Allantoic sac inoculation
3 Mode of harvesting materials containing the virus
4 Indications that infection has occurred
    a Death of embryo
    b Pathologic changes
    c Presence of hemagglutinin
    d Presence of complement-fixing antigen

B In the Monkey
1 Technic of inoculation
2 Criteria of infection
    a Swelling of parotid gland
    b Demonstration of complement-fixing antigen in the parotid gland
    c. Confirmatory serologic tests
        (1) Demonstration of complement-fixing antibody
        (2) Demonstration of antihemagglutinin
    d Tests for active immunity
    e Serial passage of virus

C In Small Experimental Animals

D On Tissue Cultures

V Certain Properties of the Egg-Adapted Virus

A Size and Shape

B Resistance to Physical and Chemical Agents

VI Diagnostic Procedures Depending upon the Demonstration of Specific Antibody

A Complement Fixation Test
1 Applications
2 Preparation and titration of antigens
    a Crude chick embryo antigens
    b Chick embryo S and V antigens
    c Monkey parotid antigens
3 Blood serum and other fluids
4 Diluent
5 Complement
6 Hemolytic system
7 The complement fixation test
8 Interpretation
    a. Complement-fixing antibody as an index of immunity
    b Complement fixation test as a diagnostic procedure

lute or relative increase in lymphocytes which may be encountered from the 1st to the 14th day of the disease. It is thus evident that the white cell count is of little importance in the specific diagnosis of infection due to this virus

2. *In spinal fluid.* In mumps meningoencephalitis, the white cell count of the spinal fluid may range from 2–3,000 leukocytes/cmm In most cases at some time during the illness the differential count characteristically reveals 90 to 100 per cent lymphocytes [8,9,10] The proportion of lymphocytes may be lower, however, in specimens taken early in the disease [10] In this connection, it should be recalled that in clinically uncomplicated parotitis as well, an increase in the white cells in the spinal fluid has often been observed Total counts entirely comparable with those encountered in frank meningoencephalitis have been recorded

Aside from the cell content, the spinal fluid in mumps encephalitis shows little significant variation from the normal The proteins and sugar may be somewhat increased, the chlorides may be slightly decreased According to Wesselhoeft,[2] the colloidal gold curve is either normal or conforms to a meningitic reaction

H Serum Amylase

The serum amylase regularly increases in mumps parotitis [11,12,13] The maximum is attained during the 1st week Thereafter it declines until normal levels are commonly reached by the 4th week. The determination of serum amylase may, therefore, be of assistance in the differentiation of mumps from other conditions giving rise to salivary gland enlargement or in the retrospective recognition of cases in which the swelling has subsided at the time of examination

II. PATHOLOGY

The pathologic changes in the salivary glands or in other organs which may be affected are not sufficiently characteristic to be of value in diagnosis even were it practicable to obtain tissues for routine examination [4]

III COLLECTION OF MATERIALS EMPLOYED IN LABORATORY DIAGNOSIS

No special precautions need usually be taken in the handling of infected materials If the worker, however, is known to be susceptible*, precautions should be taken to avoid the introduction of infected mate-

* For methods of determining susceptibility, see Sections VI, A, and VIII

C. Incubation Period

In most instances 18 to 21 days elapse between the time of exposure and the first detectable enlargement of the salivary glands

D. Period of Infectivity

The period of communicability, as determined by recent studies on the isolation of virus from the saliva in naturally occurring mumps as well as in infections experimentally induced, may extend from 6 days before signs of salivary gland involvement until 9 days thereafter.[5,6,7] The usual period of communicability, however, is probably shorter. The virus may also be present in the saliva of cases of inapparent infection and of cases of orchitis or meningitis in which enlargement of the salivary glands is absent.[7,8] Presumably, it is from such sources that cases of mumps infection may arise in which no known source of exposure can be disclosed.

E Symptoms of Uncomplicated Mumps

Uncomplicated infection of the salivary glands is manifested by enlargement of one or more of these organs—most often the parotids. The swelling in most cases reaches a maximum after 48 hours, and a gland usually remains enlarged from 7 to 10 days.

Fever of short duration and of moderate degree may be present, sometimes the rise in temperature may be negligible or absent.

F Complications

The two most common complications, orchitis (about 18 per cent) and meningoencephalitis (0 5–10 per cent) often develop between the 2d and 10th day after the onset of parotitis But these conditions may also appear either before or at the same time the salivary glands become enlarged Moreover, they may be the only presenting signs.

Ovaritis has been recorded as occurring in about 5 per cent of adult females with mumps. Other complications, such as pancreatitis and neuritis of various nerves (facial, trigeminal, optic, and auditory) as well as infections of the eye (conjunctivitis, keratitis, iritis, and retinitis) or of the inner ear are encountered more rarely.

G. White Cell Count

1. *In blood* In many cases of mumps uncomplicated by secondary bacterial infection, the total white cell count may be moderately elevated, but in others it is within normal limits, and in some it may be depressed Differentially, there is frequently, but not invariably, an abso-

remains constant It is better to ship serum than clotted blood Spinal fluid, and other body fluids such as hydrocele fluid, should be kept free of bacterial contamination and treated in the same manner as blood serum

Although in certain instances strong presumptive evidence of infection by mumps virus can be obtained from a single early specimen of serum by using V and S antigens,[18] it is essential for a final diagnosis to obtain at least 2 specimens of blood at an appropriate interval in order to demonstrate the development or increase of specific antibody. Accordingly, a blood specimen should be obtained as early as possible after the onset of symptoms, and, if only one other specimen is to be tested, again after an interval of about 14 days Usually at this time, antibody concentration is high It is preferable, however, to obtain an additional blood sample about 7 days after onset, when many individuals will have developed an appreciable titer Thus an earlier diagnosis may be made. If no antibody is demonstrated in the specimen taken on the 14th day, one should be secured on the 21st day, by which time the antibody has been found in all cases so far studied

IV. PROPAGATION OF VIRUS

A In the Embryonated Hen's Egg

The following procedures have been successfully employed in the laboratory

1. *Procedures for primary egg passage* Mumps virus from a variety of sources has been isolated in embryonated eggs These include human saliva,[6] spinal fluid,[19] blood,[20] and milk,[21] as well as the parotid gland and other tissues,[22] the parotid glands of monkeys,[23,24] and the nervous tissues of mice[25] and hamsters[26] When contaminating bacteria are known to be present, penicillin and streptomycin should be added to the material before inoculation Amounts between 500 and 1,000 units or micrograms of each per ml of inoculum can be recommended. In the preparation of saliva an equal volume of sterile infusion broth may be added and the mixture centrifuged at 3,000 r p m. for 10-15 minutes in an angle centrifuge. The addition of broth is not essential, but its inclusion probably aids in the deposition of bacteria by reducing the viscosity Antibiotics are added to the supernatant fluid after centrifugation is completed. Ten or 20 per cent suspensions of tissues are prepared by grinding them in a mortar with powdered alundum and by the grad-

rial into the mouth or respiratory tract, since a laboratory infection has occurred following the inadvertent contamination of the mouth with infected monkey gland [14] If desired, susceptible persons may be vaccinated.[15] Mumps vaccine consisting of inactivated virus in the form of infected chick allantoic fluid is commercially available

A. For Isolation of Virus from Saliva and Spinal Fluid

The virus of mumps has been repeatedly recovered from the saliva of cases with parotitis on the 1st and 2d day of the disease and occasionally thereafter It has not infrequently been isolated from the spinal fluid of cases of meningoencephalitis during a period of 6 days following onset [8] It would seem, however, desirable to collect materials as soon as possible after the appearance of symptoms

Saliva may be harvested in a suitable vessel or from small children by means of a device consisting of a thin, bent copper tube leading into a glass trap connected with an electrically driven suction pump [7] Spinal fluid is drawn in the usual manner Materials thought to contain the virus should be immediately placed in an ice bath where they may be kept for a few hours prior to inoculation For longer storage they should be distributed into pyrex tubes, which are then sealed in an oxygen-gas flame and, after freezing rapidly in a dry-ice–alcohol mixture, are placed in a dry-ice cabinet It has been determined that the addition of an equal volume of sterile, neutralized skim milk to infected-chick amniotic fluid maintains the infectivity of mumps virus unchanged for at least 1 year at about —60° C Moreover, it has been found that such virus-milk suspensions may be shipped by air to remote points without complete loss of infectivity [16] Accordingly, milk should be added to provide optimal conditions for preservation of viral activity

B For Serologic Tests

Although attempts to isolate mumps virus are warranted in special circumstances, the laboratory diagnosis of the disease is most readily accomplished, in general, by serologic methods.

Approximately 5 ml of blood are obtained The blood is taken under sterile conditions and allowed to clot firmly. The serum should then be removed as soon as possible Precautions to avoid hemolysis should be carefully observed The serum should be placed in closed containers It may be stored at 4° C for not more than 4 weeks After this period the antibody concentration in human serum may decline significantly * For this reason it is best to store sera at —10° to —15° C [17] Known positive and negative human sera to be used as controls in complement fixation tests should be preserved in this manner or in glass-sealed vials in a CO_2 ice cabinet. Under these conditions the concentration of antibody

* The antibody in monkey serum appears to be more stable

slit with tincture of iodine and 70 per cent alcohol, the egg is again placed over the candling light in an almost horizontal position The volume of inoculum is 0.1 ml By means of a sharp 1½-inch, 25-gauge hypodermic needle, the inoculation is made through the slit with a swift thrust toward the embryo A lively movement of the latter indicates that the amniotic sac has been penetrated After removal of the needle, the area of injection is again disinfected, and the opening sealed with fingernail polish.

b Yolk sac inoculation. The egg is candled to locate the boundary of the air space From the shell over the air space a triangular section with sides about 1 cm long is removed A drop of sterile mineral oil may be placed on the shell membrane at the bottom of the air space to render it more transparent The inoculum in a volume of 0 5 to 1 0 ml is then introduced into the yolk sac by means of a 1½-inch, 22-gauge needle Because of the large volume, the injection is made slowly to avoid trauma The triangular opening is then sealed with scotch tape sterilized in the autoclave The egg is incubated in a vertical position with the seal uppermost

c Allantoic sac inoculation After determining and marking the position of the embryonic eye, a slit ⅜ inch long is drilled a little to one side of the mark Through this aperture, the sac is entered, employing a ½-inch, 26-gauge needle, which is inserted at a sharp angle to the plane of the shell The position of the needle when the injection is made is almost parallel to the shell The volume of inoculum is 0 1 ml

3 *Mode of harvesting materials containing the virus* The egg is candled, the base of the air space marked, and the position of the embryo redetermined if possible Approximately ⅓ to ½ of the shell overlying the air space is removed The shell membrane at the base of the air space is carefully stripped off With the egg in an upright position and with the use of a capillary pipette, the allantoic fluid is drawn off, taking care to avoid injury to large blood vessels with consequent contamination of the fluid with erythrocytes If contamination by red cells does occur to a moderate or marked degree, the fluid is not added to the pool derived from other embryos

The egg is then placed in a horizontal position with the embryo uppermost, and the overlying shell removed. The amniotic fluid is drawn off by catching a fold of the amniotic sac with a pair of round-end forceps and by entering it with a capillary pipette. The yield of fluid from infected embryos which have been incubated for a total period of 13 to 14 days varies considerably. On the average the volume obtained

ual addition of the required quantity of suspending fluid As the latter, infusion broth or isotonic phosphate buffer solution is suitable. Spinal fluid in the crude state is used as the inoculum

For inoculation the route of choice is the amniotic sac. The usual volume of inoculum is 0 1 ml This is introduced into 6 or 8 eggs in the manner described in Section IV, A, 2, a If it is desired to make the injection under direct vision the procedure described by Leymaster and Ward[6] may be adopted. This more laborious manipulation seems unnecessary, however, provided an adequate number of eggs is employed, since it has been shown with a dye solution that the inoculum is deposited in the amniotic sac in about 90 per cent of cases by the indirect method

Seven- to 8-day embryos are selected and maintained in the manner described in Section IV, A, 2 The eggs are incubated for 5 to 7 days at 35° C after inoculation. The amniotic fluid is then removed and tested for the presence of virus (see Sections IV, A, 3, and IV, A, 4, c).

Evidence for the presence of virus may frequently be obtained in the primary passage A total of 3 serial passages using pooled mixtures of amniotic membranes and fluids should be carried out before a negative result is recorded

2 *Procedures for serial passages* Three routes of inoculation have proved most useful the amniotic sac, the allantoic sac, and the yolk sac Inoculation of the chorioallantoic membrane employing the technic of Burnet[27] has proved successful but does not appear to offer any advantage. Probably little multiplication of the virus occurs after intravenous injection since Beveridge and his associates[30] found no hemagglutinin in the amniotic fluid under these circumstances Embryos may be used which have been developed for 6 to 8 days at 39° C. The atmosphere of the incubator is humidified during this interval and may be continued during the period subsequent to inoculation, when the temperature of incubation is dropped to 35° C. The optimal post-inoculation period of incubation, irrespective of the route of inoculation, is 5 to 7 days Varying amounts of complement-fixing antigen, hemagglutinin, and infective virus are present from the 3d to the 12th day after inoculation Maximal yields can be usually obtained on or about the 5th day although the highest titers of hemagglutinin may not be attained until the 7th day.[28]

a. Amniotic sac inoculation. The egg is candled, the position of the embryonic eye located, and a slit about ⅛ inch long is drilled approximately ¾ inch above the eye over the air space. After wiping around the

the same age, the mean death rate in various lots of eggs was about 25 per cent, with a range from 11 to 43 per cent From the foregoing data, it is clear that whereas the virus is capable of killing the embryos, it does so irregularly, and therefore embryonic death cannot be regarded as a reliable index of infection

b Pathologic changes There are no gross or microscopic pathologic lesions in either the dead or living infected embryonic tissues that can be specifically associated with the effect of the virus

c Presence of hemagglutinin The demonstration of a specific hemagglutinin in materials derived from the inoculated egg may be regarded as a reliable index of infection,[24] but its absence may not necessarily indicate that infection has failed to occur In certain instances, although this factor cannot be demonstrated, complement-fixing antigen has been shown to be present in the amniotic sac The presence and concentration of hemagglutinin may be determined by the technic described in a subsequent section (VI, B, 2, a) Its specificity can be revealed by showing that hemagglutinative activity is inhibited upon the addition of specific antiserum to the system (Section VI, B, 2, b)

When hens' erythrocytes are used in the test, the highest concentration of hemagglutinin occurs in the amniotic fluid, even in material of the first egg passage, irrespective of whether the yolk sac or the amniotic sac has been selected as the route of inoculation In contrast, the factor may not develop in the allantoic fluid under these conditions of inoculation until several serial passages have been carried out Indeed with certain strains satisfactory adaptation to the allantoic system has not been obtained Yolk fluid is also hemagglutinative according to Beveridge and his co-workers [30]

Tests for hemagglutinin in amniotic membranes from earlier passages have yielded irregular results Materials from later passages have proved more regularly hemagglutinative At present, however, the routine use of amniotic membrane suspensions as hemagglutinin is not recommended

d Presence of complement-fixing antigen The method of complement fixation for determining the presence of antigen was the first to be employed as an index of infection in the embryo It is, as noted above, a somewhat more reliable procedure than the test for the presence of hemagglutinin

In the authors' experience the virus appears to have a tendency, at least in the first egg passages to multiply most regularly and actively in the amniotic membrane as indicated by the constant and high concentration of complement-fixing antigen in this tissue as compared with other embryonic constituents But after prolonged serial passage via the yolk sac and amnion or a more limited number of passages via the chorioallantoic sac, certain strains of the virus at least become adapted to the allantoic system so that titers in the same range as those obtained with amniotic material may be anticipated

B In the Monkey

1 *Technic of inoculation* The procedure for inoculation of the monkey via Stensen's duct is described in detail by Johnson and Goodpasture [31] By means of a 24-gauge hypodermic needle from which the point has been removed, the orifice of Stensen's duct is entered. This lies

is from 0.25 to 0 5 ml Larger yields are derived from eggs which have been incubated 11 to 12 days If it is desired to avoid contamination with albumin, the amniotic fluid should be harvested before the 12th day, since at this time the albumin begins to enter the amniotic sac.[29]

The remaining contents of the egg, with the exception of the chorioallantoic membrane which is retained within the eggshell by pressure with the forceps, are deposited in a sterile petri dish, taking care to separate forcibly the chorioallantoic membrane from the amniotic in the area of the point of attachment, since once the membranes are intermingled in the petri dish, it is difficult to distinguish them The amniotic membrane is then stripped from the embryo. If, during the above procedures, the embryo should have slipped out of its enveloping membrane, the membrane can be located by following along the tissue from the point of attachment to the embryo. Frequently, the amniotic membrane covers part of the surface of the yolk sac and must be carefully teased away. Because the weight of individual membranes is small, varying from 0.2 to 0.5 gm , a pool is made of 4 or 5 membranes. The tissues are washed in about 10 ml of physiologic salt solution and their wet weight determined. The yolk sac can be ruptured with the forceps in order to release the larger part of the yolk Much of the remaining yolk can be removed by washing the sac 3 or 4 times in sterile saline The wet weight of individual yolk sacs varies from 1.5 to 3 5 gms. Of both the amniotic and the yolk sacs, a 20 per cent suspension is made by grinding the material in a mortar with sterile alundum and saline. After centrifugation at about 1,500 r p m. for 10 minutes, the supernatant is preserved for further testing. Suspensions of chorioallantoic membrane and of the embryo may be prepared in a similar manner Tests for bacterial contamination are immediately carried out on each pool of material All materials can be stored in the icebox overnight until the cultures for sterility are examined. If storage is to be maintained for a prolonged period, it is best to keep the material in glass-sealed pyrex vials in the CO_2 cabinet.

4. *Indications that infection has occurred.*

a. Death of embryo The proportion of embryos which die after inocula-

(Enders strain), the death rate after yolk sac inoculation of 6- to 7-day embryos averaged 40 to 50 per cent after 7 to 9 days, although it ranged from 8 to 57 per cent in individual experiments After inoculation of the amniotic sac of embryos of

recommended for obtaining the 2d specimen of serum because the antihemagglutinin may at times appear after the complement-fixing antibody

d Tests for active immunity Johnson and Goodpasture have shown that monkeys which had recovered from mumps failed to respond with typical symptoms when reinoculated with the virus via Stensen's duct[33] The absence of typical symptoms, then, following the introduction of 2 ml of a 5 per cent suspension of parotid gland shown, by inoculation into a normal monkey, to contain active virus is evidence of active immunity resulting from the primary inoculation The development of a firm, hard swelling of the gland within 24 to 72 hours after the inoculum is administered is further evidence of the immune state This accelerated response which follows in a high proportion of resistant monkeys is in all probability a manifestation of hypersensitivity to the virus The accelerated reaction may persist for several days but is rarely discernible after the 6th day following inoculation

e Serial passage of virus The maintenance through several salivary gland passages of the virus is essential for its complete identification The gland is removed at the optimal time as defined above and tested for the presence of complement-fixing antigen The procedure should be repeated until typical swelling and edema are regularly produced

C In Small Experimental Animals

Mumps virus has been reported to multiply in the parotid glands of cats,[34] but this has not been confirmed More recently mumps virus has been shown to multiply and cause corneal opacity after injection into the anterior chamber of the guinea pig's eye [35,36] and to grow in the lungs of adult hamsters after intranasal inoculation [37] The latter method has been applied to the demonstration of virus in saliva from mumps patients by taking an antibody response in the hamster as the criterion of virus multiplication Egg-adapted mumps virus has been successfully adapted to the brains of suckling hamsters and suckling mice by Kilham and his associates, who also reported similar adaptation directly from certain infected human materials [38,38a] These adapted strains in very young animals produce a fatal encephalitis which may be prevented in a neutralization test by convalescent mumps serum

D. In Tissue Cultures

Henle and Deinhardt[38a] have recently shown that cultures of the HeLa strain of human carcinoma cells and of trypsinized monkey kidney cells can be effectively applied to the isolation of mumps virus from the saliva. The results of a comparative study demonstrated that the tissue culture reveals the presence of virus more frequently and more rapidly than the chick embryo HeLa cell cultures proved more satisfactory than those of monkey renal cells since cytopathic changes induced by the

adjacent to the papilla situated on the buccal mucous membrane, at a point approximately opposite the 1st upper molar. About 2 ml. of the crude diluted saliva is gently forced into the gland. Visible swelling of the gland or its increase in size as revealed on palpation during the process of inoculation will indicate whether or not the material has been successfully introduced. The bacteria that contaminate saliva rarely persist in the gland or cause an infection

2 *Criteria of infection* Although some animals may respond to infection by a rise of temperature and leukopenia, these manifestations have not been constant features of the experimental disease Dependence is placed upon the fulfillment of two or more of the following criteria of infection In most instances, those mentioned under a, b, and c have been regarded as sufficient evidence for the nature of the disease

a Swelling of parotid gland Enlargement of the inoculated parotid gland due to the infection is not observed in normal monkeys until the 6th or 7th day following the introduction of virus Thereafter, in typical cases, increase in size of the gland occurs rapidly, accompanied by marked edema of the surrounding tissues These manifestations, in most instances, reach their height on the 8th or 9th day and then rapidly subside The degree of enlargement of the gland after inoculation of saliva may be minimal and may be accompanied by little or no edema On subsequent passages, however, definite swelling of varying degrees is a constant feature

b Demonstration of complement-fixing antigen in the parotid gland Complement-fixing antigen is nearly always present and in maximal concentration in the parotid gland on the 5th to the 6th day after inoculation Its demonstration, therefore, is a reliable criterion of infection

Infected gland has proved unsatisfactory as a source of hemagglutinin since it has not exhibited the capacity to agglutinate most lots of chicken erythrocytes, which are, however, agglutinated by infected egg fluid

c Confirmatory serologic tests

(1) Demonstration of complement-fixing antibody Complement-fixing antibody appears in all animals inoculated with active virus and tested between the 10th and 14th day For its demonstration, a blood sample should be taken at the time of inoculation and at least once again on about the 14th day, when titers corresponding to those found in convalescent human serum should be encountered if the inoculum contained virus (see Section VI, A, 7) Specimens of serum are obtained before inoculation because monkeys in contact with others in the acute stage of mumps may undergo an inapparent disease that results in the development of complement-fixing antibody

(2) Demonstration of antihemagglutinin A serum factor which inhibits the agglutination of red cells by the virus likewise develops regularly following infection [32] A sample of serum should be obtained before inoculation and again on about the 18th day thereafter, and tests for the inhibiting antibody should be carried out according to the technic described in Section VI, B, 2 The 18th day is

VI. DIAGNOSTIC PROCEDURES DEPENDING UPON THE DEMONSTRATION OF SPECIFIC ANTIBODY

A. Complement Fixation Test

1 *Applications* The complement fixation test has proved to be a useful tool in the laboratory The following is a list of circumstances in which this test has been employed

a For the determination of immunity in an individual exposed to mumps [41,42]

b To distinguish, from a group of potentially susceptible persons, the large number that would be resistant to the disease—of use in experiments for the evaluation of prophylactic agents, since about one-third of the population has undergone an inapparent infection and is presumably immune [43,44]

c To observe the effects of vaccination in man and monkey [17,43,44,45]

d To diagnose mumps meningoencephalitis, particularly in the absence of involvement of the salivary glands [8,9,10,46]

e To distinguish other salivary gland diseases from mumps [47]

f To detect the multiplication of virus in the parotid gland of the monkey that has been inoculated [17]

g To detect the multiplication of virus in the various tissues of embryonated eggs [23 24 30]

2 *Preparation and titration of antigens.* The following antigens may be used in the complement fixation test

Crude chick embryo antigens
Chick embryo S and V antigens
Extract of parotid glands of infected monkeys

a Crude antigens derived from infected chick embryos Allantoic or amniotic fluid from this source is now generally used as complement-fixing antigen Since allantoic fluid may be obtained in larger amounts, it has been most extensively employed These materials are harvested on the 5th or 6th day from embryos previously inoculated with mumps virus by the appropriate route. Their capacity to fix complement in the presence of either monkey or human serum known to contain antibody (convalescent serum) is determined in a "box" or "checkerboard" titration. Increasing dilutions of the fluid (undiluted through 1:64) are tested against suitable dilutions of serum (e g, 1.8 through at least two dilutions beyond the serum end point if this is known from previous determinations with other lots of antigen If the serum end point is unknown this should be approximately determined, using infected fluid diluted 1:2) The highest dilution of antigen that gives the highest serum titer is used in subsequent tests. Each antigen should be titrated against at least 2 positive sera as well as 2 or more sera known to contain

mumps virus became evident sooner in the former and were not to be confused with those produced by so-called "foamy viruses" often latent in monkey cells It would seem, therefore, that the tissue culture may offer in the future the method of choice for the isolation of mumps virus At present its chief disadvantage as compared with the chick embryo lies in the fact that viral hemagglutinin does not usually appear in the culture fluid The absence of hemagglutinin makes is necessary to carry out in vitro neutralizing tests, which are laborious and time-consuming

V. CERTAIN PROPERTIES OF THE EGG-ADAPTED VIRUS

A. Size and Shape

Johnson and Goodpasture[31] demonstrated that monkey parotid virus will pass Berkefeld N and V filters[31] With the Elford graded-porosity membranes, Enders[38] found the egg-adapted virus to measure 90 to 135 mμ whereas Habel[23] found it to be over 340 mμ Electron micrographs have indicated about 233 mμ[28] with considerable variation in size and irregularities of shape In the same study the sedimentation diagrams showed two boundaries—a principal one with a constant $S = 1311 \times 10^{-13}$ and a secondary peak at $S = 1940 \times 10^{-13}$.

Other studies[18] have revealed the ease with which concentration of the infective virus particles may be obtained by centrifugation (20,000 r p m for 20 minutes) To sediment the soluble complement-fixing antigen, however, requires a greater force applied for a longer period (30,000 r p m for 1 hour).

B Resistance to Physical and Chemical Agents

Most of the properties of mumps virus are relatively resistant to heat, complement-fixing antigen withstanding 80° C for 20 minutes Infectivity and hemagglutinin may be destroyed at 55° C for 20 minutes,[39] but Kilham[40] has found with certain strains a greater stability of both these properties at 56° C At room temperature he was able to demonstrate infectivity and hemagglutination after 91 days in the case of certain recently isolated strains All the properties of mumps virus, including infectivity, will be preserved for at least 1 year if kept at —50° C to 70° C When the protein content of the suspensions is low as in infected amniotic or allantoic fluid, it is desirable to augment it by the addition of an equal volume of sterile, defatted, neutralized milk Otherwise, marked loss in infectivity may occur after a few months The activity of the virus in egg fluids may also be preserved during transit by air mail by the addition of sterile milk without further precautions At 4° C the hemagglutinin, complement-fixing and skin test antigens will remain potent for several months

Mumps virus has been reported to be most stable in the pH range of 5 8 to 8 0.[28] Infectivity is readily destroyed by treatment with 0 2 per cent formalin at 4° C. [illegible] complement-fixing antigens This [illegible] The infectivity is also destroyed

antigen This is prepared[17] by grinding the tissue with powdered alundum in a mortar with the gradual addition of physiologic salt solution until a 20 per cent suspension is attained The suspension is then centrifuged at 3,500 r p m for 30 minutes in an angle centrifuge The opalescent fluid is removed from the sediment, taking care *not* to include the fatty pellicle at the surface. The antigen thus obtained is titrated in exactly the same manner as described for chick embryo materials except that a wider range of dilutions are employed (1 20 through 1.2,560) and an extract of normal monkey parotid tissue prepared in the same manner is included as an additional control of the specificity instead of normal embryonic materials

3 *Blood serum and other fluids* Human serum is heated at 60° C, monkey serum at 62° C., for 20 minutes just before the tests are carried out If the serum is found to be anticomplementary, heating for a second time, after allowing the serum to stand at 4° C or even room temperature for an hour, will usually remove this property without affecting the antibody content[17] The second heating may also be carried out after the serum is diluted and stored in the icebox overnight

Other fluids such as spinal fluid and hydrocele fluid may be heated at 60° C for 20 minutes just before the test is performed to remove any anticomplementary activity as well as to inactivate any complement that may be present

4 *Diluent* Veronal buffer, containing quantities of Ca++ and Mg++ optimal for complement activity, is used as diluent for all reagents.[49]

5 *Complement* Sera from at least 6 guinea pigs are collected, pooled, and stored in glass-sealed ampules in the CO_2 cabinet No diminution of complement titer has been observed under these conditions over a period of several months For this reason, it is not necessary to titrate the complement each day a test is run It is desirable, however, to check the titer of the complement whenever new lots of reagents (sheep cells or amboceptor) are being used for the first time

Complement titrations are carried out in duplicate, using a stock solution of freshly thawed guinea pig serum diluted 1.10 One ml of this solution is further diluted to 1.90 and volumes of 0 04 ml., 0 06 ml., 0 08 ml, 0 10 ml, 0 12 ml, 0 14 ml, 0.16 ml, 0.18 ml, and 0 20 ml are pipetted into a series of tubes. (It may be necessary to shift this range of volumes with different lots of complement.) These volumes are in-

no mumps complement-fixing antibodies. The quantities of reagents, the controls, and the procedures are the same as those described below for the complement fixation test itself. The end point of reaction is considered to be the highest dilution in which 3+ or 4+ fixation occurs As additional controls of the specificity, allantoic or amniotic fluid from normal embryos of the same age or a 20 per cent extract in salt solution of the allantoic and amniotic membrane from the same source are employed All complement-fixing antigens should preferably be stored in the CO_2 cabinet until wanted for use.

b. S and V antigens prepared from infected chick embryos The use of purified S and V antigens in certain instances has been reported as being especially helpful in the serologic diagnosis of mumps virus infections [18,46,48] The preparation of these antigens was described as follows by the Henles [48]

Suitable numbers of 8-day-old chick embryos were inoculated with 0 5 ml of infected allantoic fluids diluted in broth to 10^{-2} to 10^{-4} by the usual technic After 5 days of further incubation of the eggs at 36–37° C the allantoic fluids as well as the allantoic sacs were harvested aseptically The fluids containing the virus-bound antigen (V) were dialysed in sterile cellophane bags against 20 volumes of M/100 phosphate-buffered saline of pH 7 0 in order to remove most of the urates prior to irradiation by a technic previously described [48a]

The sacs were thoroughly washed in sterile buffered saline solution, drained on sterile filter paper, and weighed A 20 per cent suspension in buffered saline solution was made by emulsifying the tissue in a Waring blendor for 3 minutes After preliminary centrifugation of the suspension at 2,000 r p m for 10 minutes, the supernatant fluid was subjected to high speed centrifugation at 20,000 r p m. for 20 minutes The supernatant fluid obtained after this centrifugation served as soluble (S) antigen

Control antigens were prepared according to the method described above from uninfected chick embryos of the same age and usually from the same batch of eggs supplying the mumps preparations The normal allantoic fluid gave positive complement fixation tests with human sera only very rarely In these cases, the control antigens prepared from normal allantoic membranes, likewise, gave positive results The normal allantoic fluid was omitted, therefore, in later tests

The dialysed infected allantoic fluid was irradiated in order to inactivate the virus, and the membrane antigen was treated in the same way To all antigens 1 10,000 merthiolate was added as a preservative They proved to be stable at 4° C for at least 3 months

c Monkey parotid antigens. For special purposes (e g , to investigate the cause of the rare false positive reactions that may occur with certain human or monkey sera and antigens derived from chick embryos), it may be desirable to employ an extract of infected monkey gland as the

c. Unknown serum (lowest dilution) and 1 unit of complement

d Unknown serum (lowest dilution), 2 units of complement and normal antigen (normal parotid gland suspension or normal embryonic fluid or membrane suspension)

e Complement activity controls consisting of duplicate series of 4 tubes containing ½, 1, 1½, and 2 units of complement, respectively

f Controls for anticomplementary activity of all antigens employed, to each antigen is added 1 unit and 2 units of complement, respectively

In addition a number of dilutions of a known positive serum are prepared sufficient to cover the end point range To these are added test antigen and 2 units of complement Controls for the anticomplementary effect of this positive serum and its nonspecific reaction with normal antigen are included (see controls 2, 3, 4 above) *A known negative serum diluted 1 2, test antigen, and 2 units of complement* with the usual controls for this serum should also be included

After the addition of salt solution sufficient to bring the volumes to equivalence with those of the test, the control tubes are maintained under the same conditions and to each is added 0 25 ml of sensitized sheep cells the following morning

8 *Interpretation* a Complement-fixing antibody as an index of immunity The available data[41 42] indicate that when this antibody is present in human sera it has arisen as the result of previous apparent or inapparent infection except in certain persons who may have received mumps vaccine or the skin test antigen[43,51] Moreover, there is no evidence that in other acute infections the antibody may appear or increase as a result of an anamnestic response Hence, because of the solid and enduring immunity in mumps, the presence of complement-fixing antibody may be regarded with a high degree of probability as an indication that the individual is resistant Direct evidence[41,42] for the validity of this interpretation is afforded by the fact that the number of cases of mumps occurring in positive reactors has been very low (1 to 2 per cent) Failure to demonstrate complement-fixing does not, however, exclude the possibility of previous infection and hence presumptive immunity since it has been found that 20 to 30 per cent of those giving a positive history of mumps fail to give positive tests[41,42] The accuracy with which the immune status of an individual can be evaluated is increased by the concomitant use of the skin test (see Section VI, B) If both tests are positive the chances of acquiring an infection following natural exposure appear to be exceedingly small[42]

A proportion of the small number of false positive complement fixation reactions can probably be attributed to materials appearing in the fluids as a result of infection of the chick embryo but quite unrelated to the virus Such materials presumably react with an antibody or antibodylike factor in the serum of certain individuals Thus it has been shown that the serum of at least 1 individual that fixed complement in the presence of infected allantoic fluid failed to react with antigen prepared from infected monkey parotid gland This serum exhibited no fixation with normal allantoic fluid or membrane extract

b Complement fixation test as a diagnostic procedure Conclusive serologic evidence of infection by the virus of mumps consists in the demonstration of a 4 fold or *greater increase in the concentration of complement-fixing antibody during*

creased to 0.5 ml. by the addition of veronal buffer, and 0.25 ml. of sensitized cells (see 6 below) is added. The contents of the tubes are mixed and incubated in the water bath at 37° C for 30 minutes The tubes are agitated at 5- to 10-minute intervals to keep the cells in suspension. The unit of complement is taken as the smallest amount that gives complete hemolysis in the titration For use in the test the complement is further diluted so that 0.3 ml contains 2 units

Because of the presence of optimal amounts of Ca^{++} and Mg^{++} in the diluent, the antigens described above usually have neither an enhancing nor inhibitory effect on the complement, and it is therefore seldom necessary to titrate the complement in the presence of the antigens The controls included in each test (see 7 below) will indicate relative amount of residual free complement present at the completion of the test.

6. *Hemolytic system* Sheep blood collected in modified Alsever's solution[50] can be stored for two months or more in the refrigerator and still serve as a source of red cells for the hemolytic system. For use in the test the cells are washed 3 times in veronal buffer and a 2 per cent suspension prepared To 1 volume of this suspension, 1 volume of diluted antisheep serum containing 2 units of amboceptor is added 15 minutes before the sensitized cells are used in the test

7. *The complement fixation test.* Twofold dilutions in buffer of the inactivated serum to be tested are prepared in appropriate volumes, taking care that the lower dilutions are in sufficient quantities to satisfy the various controls that will be required For diagnostic tests, dilutions of 1:2 through 1:250 usually are sufficient Into each of a series of tubes, 0.1 ml. of each dilution is then pipetted To each tube are added 2 units of complement in a volume of 0.3 ml., and then 0.1 ml. of appropriately diluted antigen The mixtures are shaken and kept overnight at 4° C. To each are added 0.25 ml. of sensitized sheep cells after all reagents have come to room temperature the following morning The degree of hemolysis is recorded after incubation for ½ hour at 37° C. in the water bath. The end point is taken as the highest dilution giving definite fixation of complement denoted as 4+ or 3+ Complete fixation is denoted at 4+.

complement

a Titration of hemagglutinin A series of 2-fold dilutions of the infected fluid (amniotic or allantoic) are prepared in 0 85 per cent sodium chloride solution. A separate pipette is used for each dilution To 0 5 ml of the diluted fluid in 8 mm round-bottomed tubes is then added 0 5 ml. of 0 25 per cent suspension of pooled erythrocytes (from 4 chickens) that have been washed 4 times in isotonic salt solution After thorough mixing the tubes are allowed to stand for approximately 1 hour at room temperature when the degree of agglutination in each tube is recorded according to the cell pattern formed on the bottom of the tube (see Section VI, B, b, 3) The highest final dilution of the fluid that gives complete agglutination is considered to contain 1 hemagglutinating unit per ml

b Titration of antihemagglutinin Sera to be tested are inactivated at 56° C for 30 minutes and serial 2-fold dilutions prepared in salt solution To 0.25 ml of each dilution of serum is then added 0 5 ml of diluted embryonic fluid containing 8 hemagglutinating units of virus per ml , thus a total of 4 units is used in the test

The following controls are included·

(1) 0 25 ml of lowest dilution of serum tested + 0 5 ml salt solution

(2) 0 5 ml. of diluted embryonic fluid (4 units) + 0 25 ml. salt solution

(3) Appropriate dilutions of acute and convalescent phase mumps sera known to present at least a 4-fold difference between their titers.

Control 1 reveals any modifying effect the serum itself may have on the pattern of agglutinated cells Control 2 confirms the hemagglutinating activity of the virus Control 3 confirms the specificity of the results

The test mixtures and the controls are kept at 37° C for 1 hour. At the end of this reaction period, 0 25 ml of 0 5 per cent suspension of washed chicken erythrocytes is added to each tube After thorough mixing, the preparations are allowed to stand at room temperature for about 1 hour The clearest readings can be made just before all the cells have settled on the bottom of the tube This condition is obtained after about 1 hour at ordinary room temperature (18–20° C.). If the temperature is unusually elevated or depressed the rate of settling will be shorter or longer since it has been found to vary with the temperature If the cells are allowed to stand until complete settling has occurred, the characteristic, evenly distributed layer of agglutinated cells often shrinks together, forming a pattern which may simulate that of the round "but-

the course of the disease * In parotitis the antibody first appears in many individuals on the 5th to the 7th day following the appearance of the swelling and is to be found in nearly all cases by the end of the 2d week Occasionally, antibody may be demonstrated at the time of the onset In other manifestations of infection, particularly meningoencephalitis, antibody may have attained a high level by the time the first specimen of serum is obtained, and no increase in titer can be demonstrated, especially when a single antigen is used in the test Under these circumstances, however, titers of 1 64 or higher are "suggestive" of the nature of the infection since they are only rarely encountered in the sera of individuals who have experienced an infection in the more or less remote past Presumptive evidence of infection may be obtained in a certain proportion of cases early in the disease if both S and V antigens are employed since, as the Henles have shown,[48] the S antibody frequently tends to reach its maximum more rapidly Thus if a high anti-S and a low anti-V titer are encountered in the first specimen of serum taken, a presumptive serologic diagnosis may be made at that time If a significant increase in the V antibody can be subsequently determined, the serologic diagnosis is rendered conclusive

In cases where it is desired to invoke the complement fixation test as a diagnostic aid, skin-testing should not be done since it may be followed by the formation of specific antibody or by its increase if already present

B Inhibition of Hemagglutination

1 *Applications* So far, the inhibition of hemagglutination by serum containing specific antibody has been employed in the following circumstances·

a To demonstrate specificity of the hemagglutination observed in the fluids of the infected egg Several viral agents[56,57,58,59] as well as other factors[60,61] can cause agglutination of erythrocytes indistinguishable from that brought about by the fluids from chick embryos infected with the mumps virus

b To demonstrate specific antihemagglutinin for diagnostic purpose in persons recently convalescent from mumps[62,63,64]

c. To measure the immune response following vaccination with inactivated and attenuated mumps vaccines[44,65]

2 *Technic.* The methods of Hirst[56] and of Salk[66] have been employed. In most recent investigations that of Salk with certain modifications has been adopted. The details of the test as given below are taken from the paper by Robbins and his associates.[62]

the test *in ovo* utilizes an animal—the chicken embryo—which shows no visible or obvious sign of its mumps infection, so that virus neutralization by the serum under test must be demonstrated by indirect means. Lack of virus multiplication in the embryonated egg is the criterion of neutralization, and this may be shown either by complement fixation of pooled amniotic or allantoic fluids against a known mumps antiserum or more easily by testing for the presence of viral hemagglutinin This latter procedure has recently been standardized by Gotlieb and her co-workers[67] and with slight modification is as follows

A pool of mumps-infected allantoic fluid should be made and stored at —50° C to —70° C in sealed ampules after shell freezing The infectivity titer of this pool for 8-day-old chick embryos should be determined under the conditions of the actual test as given below Sera should be inactivated at 60° C for 20 minutes and preferably stored at —20° C until tested Serial 2-fold dilutions of sera in physiological saline are made in 0 5 ml volumes and to these are added equal volumes of the standard virus diluted in beef heart infusion broth to contain 1,000 to 10,000 ID_{50} per egg dose As a precaution against bacterial contamination from serum specimens, it is usually advisable to add 250 units each of penicillin and streptomycin per ml to the virus-serum mixtures before incubation Incubation may be for 1 hour at 37° C or, if more convenient, at 4° C for 18 hours At the end of this period each mixture is inoculated in amounts of 0 2 ml into the allantoic sac of six 8-day-old chick embryos After incubation of these eggs at 36° C for 6 to 7 days, individual allantoic fluids are harvested and checked for the presence of hemagglutinin by adding a 1 per cent suspension of chicken erythrocytes In each test a standard known positive serum at an intermediate dilution should be included along with a titration of the test virus, employing serial 10-fold dilutions to which equal volumes [illegible]

Factors influencing the above standardized procedure have been evaluated[67] The potentiating effect of a heat-labile component in fresh sera upon neutralizing capacity of serum as reported by Leymaster and Ward[68] appeared to be mediated through its inhibitory effect on virus titer and was only minimal in the actual neutralization test. This effect is eliminated by inactivation of all sera before use in the test. In correlating the quantity of virus with the quantity of antibody, Gotlieb and her associates present good evidence that it requires a 1,000-fold decrease in the amount of virus used before a 10-fold increase in neutralization index of a given serum will take place This gives very reasonable practical limits within which standard test virus titers may vary without appreciable alteration in serum indices. The most significant variable is due to the irregularities of viral multiplication in individual eggs when

ton" presented by cells not agglutinated by the virus. As a rule, no difficulty is experienced in reading the tests except in tubes containing the largest amounts of serum In these the pattern is often atypical, consisting of a broad flat button usually with an irregular periphery. This is regarded as the effect of partial agglutination, probably modified by the presence of serum. It can be readily distinguished from a true negative reaction, which is manifested by a small, round discrete button of cells that streams when the tubes are tilted. The atypical button does not stream but, remaining intact, may slide down toward the lower side of the tube. Further aid in interpreting these atypical reactions may be obtained by comparing them with the appearance of the cells in control No 1 These reactions are without doubt attributable to nonspecific antihemagglutinating factors present in normal serum. Certain workers have found it desirable to reduce their effect by treating the sera with $NaIO_4$ For this purpose Henle and her co-workers [42] have satisfactorily employed the following technic: 0 15 ml. of 0.1M $NaIO_4$ is added to 0.5 ml of the serum The mixture is allowed to stand for 30 minutes at 37° C. when 0.15 ml of 40 per cent glucose solution is added The volume is then brought to 1 ml by the addition of salt solution

The antihemagglutinin titer is expressed as the reciprocal of the final dilution of serum, which completely prevents agglutination of the cells by the virus

3 *Interpretation* The available data [62,63,64,65] indicate that a 4-fold or greater rise in antihemagglutinin affords conclusive evidence of infection by the mumps virus (see footnote, Section VI, A, 8, b) If the first specimen of serum is taken within 4 days after the onset, a significant increase in this antibody can be demonstrated in nearly all cases, if taken between 4 and 8 days, an increase cannot always be detected since the maximal level may already have been attained In general the results of comparative tests have shown that those obtained by the hemagglutination inhibition test closely parallel those obtained by the complement fixation technic In a few instances, however, it has been possible to demonstrate a significant increase by one method and not by the other Since the hemagglutination inhibition test is easier to perform and the results are more rapidly available, it would appear to be the routine procedure of choice

The antihemagglutinin test has not been applied successfully as a means of determining resistance or susceptibility because of the lack of methods for eliminating with certainty all nonspecific factors in serum that may inhibit hemagglutination

C. Virus Neutralization Test

1. *In the embryonated egg* Virus neutralizing antibodies may be demonstrated in mumps immune or convalescent sera by technics essentially the same as those used in neutralization of other viruses However,

the same type of response. During the past several years, mumps skin test antigen and control material prepared from infected chick embryo allantoic fluid has been available commercially in the United States *

A Preparation of Antigens

Although most of the early published findings with the mumps skin test were obtained by the use of material prepared from infected monkey parotid gland tissue, at the present time egg-adapted virus is the most practical source of skin test antigen. For this reason the method of preparation will be given for chick embryo virus, although it is equally applicable to emulsions of infected monkey parotid gland.

Allantoic fluid of high virus titer prepared by usual technics (see Section IV, A, 2) is used as the source of antigen The titer of egg infectivity should probably be above 10^{-6} The infected fluid itself undiluted or diluted with phosphate buffer solution is inactivated by heating in a water bath to 65° C for 20 minutes A preservative such as 0 5 per cent phenol or 1 10,000 merthiolate is then added After storage at 4° C for several weeks the material is either filtered or centrifuged to remove any precipitate which tends to form on standing in the cold Some laboratories prefer to inactivate by exposure to ultraviolet light[42] or to concentrate and purify the virus antigen from the allantoic fluid by alcohol precipitation in the cold (25 per cent ethyl alcohol at 0° C) Another procedure recommended for purposes of purification consists in the dialysis of the heated infected allantoic fluid before the addition of a preservative [42]

Tests required before use of any skin test material in human subjects include those for sterility, lack of infectivity for chick embryo as a check on inactivation, general safety as indicated by lack of effect of inoculating mice with 0 5 ml intraperitoneally, and finally a test of potency Potency apparently can be based on the quantity of complement-fixing antigen contained in the material, 4 units of antigen (at least a 1 4 dilution of final skin test material should give a 4+ fixation in presence of known positive serum) having been found to be potent in actual use Further control of potency may be obtained by a trial screening test on several human subjects with a known positive history of mumps.

Control material for use in the test consists of normal chick embryo

* Eli Lilly, Lederle Philadelphia Serum Exchange

hemagglutination is used as the criterion of infectivity. The recommended high infectivity of the test virus and longer periods of incubation tend to reduce this variability. The use of pools of fluid from all eggs inoculated with any one dilution of serum and the complement fixation test for demonstrating virus appeared to reduce this irregularity but also seemed to give lower neutralizing indices for convalescent mumps sera [69]

2. *In suckling mice and hamsters.* Kilham *et al.*[70] have described a direct neutralization test in suckling mice using a mouse or hamster brain-passage virus Their results were comparable to those obtained by duplicate tests *in ovo*. The technic involves the admixture of inactivated serum dilutions with 100 LD_{50} of suckling mouse or suckling hamster brain-passage virus, incubation at room temperature for 30 minutes, then intracerebral inoculation of 8 randomly distributed 1-day-old Swiss mice Symptoms of encephalitis appear after the 9th day, and death is taken as the basis for the calculation of 50 per cent end points Mice are held for a total of 3 weeks before the test is terminated Mumps virus well adapted to suckling animals is required for this test, but there is the advantage of definite criteria of infection and direct test for the presence of antibodies in the sera.

3 *Interpretation* a Neutralization test as an index of immunity. Accumulating evidence in two different investigations indicates that the presence of neutralizing antibodies in human sera even if the index is as low as 2 signifies past experience with the mumps virus and probable immunity [15,71] In 767 individuals previously negative for the presence of neutralizing antibodies by an *in ovo* technic at a 1 2 dilution of serum, Habel[15] recorded 68 cases of mumps during an epidemic, whereas in 420 whose serum was positive only 2 cases of clinical disease subsequently developed in spite of approximately equal exposure Evidence presented in the reports just mentioned also strongly suggests that neutralizing antibody response to mumps vaccine is the best indication of effective immunization

b Neutralization as a diagnostic procedure Although neutralizing antibodies are readily demonstrable by the end of the 1st week in mumps infection and reach a peak at 3 weeks,[67,69,72] the *in ovo* test is too cumbersome to be used for routine diagnostic purposes Perhaps the direct neutralization in suckling mice might be more practical, especially in those laboratories not equipped to do in vitro serologic tests

VII SKIN TEST

Enders[73] was the first to demonstrate that human beings immune to mumps reacted to an intradermal inoculation of inactivated virus from the infected monkey parotid gland. Subsequently, Habel[23] showed that chick embryo adapted mumps virus after similar inactivation elicited

associates[74] have reported that the attack rate was 23 per cent in 340 negative reactors (monkey parotid antigen) subsequently exposed to mumps The Henles[42] found 1 per cent of 1,028 skin test positives, 5 per cent of 236 with doubtful reactions and 19 per cent of 614 negatives subsequently developed mumps. Thus, as has been found by other investigators,[75,76] a positive skin test is not absolute but very strong evidence of actual immunity to mumps

The skin test finds its chief usefulness in the determination of the immune status It is of little value in the diagnosis of suspected mumps infections A negative history of mumps, especially in an adult, does not necessarily indicate that the individual is susceptible because as many as 60 per cent of these individuals may have been immunized as a result of a previous inapparent infection[73] When, therefore, a person with a negative history is exposed, the question arises as to their likelihood of getting the disease and in consequence the advisability of attempting specific prophylaxis with immune serum A positive skin test under these circumstances can relieve anxiety and eliminate consideration of serum prophylaxis In the use of mumps vaccine for active immunization the skin test affords a convenient method of selecting those presumably susceptible to the disease

Theoretically, the skin test could be used diagnostically since a positive test at the onset of the suspected illness should rule out mumps Enders and co-workers[14] had found that with monkey parotid antigen the skin test often did not become positive until a week or more after onset, but Habel,[69] using chick embryo antigen, found that in uncomplicated mumps in adult males the skin test was frequently positive during the first 5 days of illness In fact he suggests that a skin test done at the onset of mumps might be a good indicator of whether or not a given case is likely to develop a complication such as orchitis

It should be noted that in some individuals who have no demonstrable antibodies prior to the skin test, they may subsequently develop This phenomenon is mentioned more often with positive skin tests Likewise persons who already possess serum antibodies will often respond to a skin test with a rise in titer[74 75]

VIII REMARKS ON THE USE OF DIAGNOSTIC PROCEDURES IN MUMPS

In mumps infections, as in many diseases, the direct isolation and identification of the virus itself from acutely ill patients affords the most satisfactory evidence for the nature of the etiologic agent. Though in mumps the isolation of virus by amniotic sac inoculation of chick embryos and its identification by the hemagglutination inhibition technic are relatively simple procedures, nevertheless they do not lend themselves to routine application in a diagnostic laboratory. Furthermore, negative results are meaningless

Indirect serologic tests in vitro are fortunately quite adequate for the specific diagnosis of mumps infections. In the diagnosis of acute disease, whether this may consist of the typical parotitis or the atypical forms such as meningoencephalitis, orchitis, or pancreatitis in the ab-

allantoic fluid which is carried through the identical procedures followed in preparing the antigens, including the various safety tests outlined above

The life of the potency of the skin test antigen is considered by the commercial companies to be 18 months from the date of issue if kept at 0°–4° C.

B. Technic of the Test

After cleaning the midflexor surface of the forearm with alcohol or acetone, 0 1 ml. of the control material is inoculated intradermally in the left arm and the mumps antigen in the right arm Before performing this test the simple question: "Are you able to eat eggs without any difficulty?" will rule out severe allergy to eggs. Those giving a history of egg allergy should not be subjected to the test

The results of the test should be read at 24 hours and again between 36 and 48 hours If only one reading is possible it should be taken at about 36 hours. Evidence of a positive reaction disappears rapidly after 48 hours. Erythema and edema are measured and recorded

In a few hypersensitive individuals areas of erythema 8 or 10 cm in diameter may be seen, but the usual positive reaction will average 2 cm In most tests which will eventually be definitely positive the reaction is already apparent at 24 hours. The peak of erythema and edema, however, usually occurs between 36 and 48 hours. In the majority of cases the zone of erythema is prominent, with well-demarcated outline. In a small proportion of positive reactions the skin is only slightly pinkish or red in color. Induration is not marked as a rule although in most instances easily recognizable Even in exceptionally extensive reactions necrosis of the skin has not been observed

Inoculation of the control material is essential because not infrequently individuals with a negative history for allergy to eggs will exhibit a positive skin reaction to normal egg material Usually, this *reaction attains its maximum by 24 hours and diminishes* or disappears by 48 hours. Many people will react to the control material but the area of erythema will usually measure less than 1 cm If the control reaction measures over 1 cm the result is difficult to evaluate. Reactions to control antigen are less frequent with monkey parotid antigens.

C. Interpretation

Evidence indicates that most reactions of 10 mm diameter or larger indicate immunity to mumps although some prefer to use 15 mm as the minimal positive reaction and regard those between 10 and 15 mm as doubtful [42] Enders and his

5. Gordon, J E, and Kilham, L Ten Years in the Epidemiology of Mumps *Am J M Sc*, 218 338–359, 1949

6 Leymaster, G, R, and Ward, T G Direct Isolation of Mumps Virus in Chick Embryos *Proc. Soc Exper Biol and Med*, 65 346–348, 1947

7 Henle, G, Henle, W, Wendell, K K, and Rosenberg, P Isolation of Mumps Virus from Human Beings with Induced Apparent or Inapparent Infections *J Exper Med*, 88 223–232, 1948

8 Kilham, L Mumps Meningoencephalitis with and without Parotitis *Am J Dis Child*, 78 324–333, 1949

9 Kane, L W, and Enders, J F Immunity in Mumps III The Complement Fixation Test as an Aid in the Diagnosis of Mumps Meningoencephalitis *J Exper Med*, 81 137–150, 1945

10 Kravis, L P, Sigel, M M, and Henle, G Mumps Meningoencephalitis with Special Reference to the Use of the Complement Fixation Test in Diagnosis *Pediatrics*, 8 204–215, 1951

11 Applebaum, I L Serum Amylase in Mumps *Ann Int Med*, 21 35–43, 1944

12 Candel, S, and Wheelock, M C Serum Amylase and Serum Lipase in Mumps *Ann Int Med*, 25 88–96, 1946

13 Wolman, I J, Evans B, Lasker, S, and Jaegge, K Amylase Levels during Mumps, Findings in Blood and Saliva *Am J M Sc*, 213 477–481, 1947

14 Enders, J F, Cohen, S, and Kane, L W Immunity in Mumps II The Development of Complement-Fixing Antibody and Dermal Hypersensitivity in Human Beings Following Mumps *J Exper Med*, 81 119–135, 1945

15 Habel, K Vaccination of Human Beings against Mumps Vaccine Administered at the Start of an Epidemic I Incidence and Severity of Mumps in Vaccinated and Control Groups *Am J Hyg*, 54 295–311, 1951

16 Enders, J F Unpublished data

17 Enders, J F, Kane, L W, Cohen S and Levens J H Immunity in Mumps I Experiments with Monkeys (*Macacus mulatta*) The Development of Complement-Fixing Antibody Following Infection and Experiments on Immunization by Means of Inactivated Virus and Convalescent Human Serum *J Exper Med*, 81 93–117, 1945

18 Henle, G, Henle, W and Harris, S The Serological Differentiation of Mumps Complement-Fixation Antigens *Proc Soc Exper Biol and Med*, 64 290–295, 1947

19 Henle, G, and McDougall, C L Mumps Meningo-Encephalitis Isolation in Chick Embryos of Virus from Spinal Fluid of a Patient. *Proc Soc Exper Biol and Med*, 66 209–211, 1947

20 Kilham, L Isolation of Mumps Virus from the Blood of a Patient *Proc Soc Exper Biol and Med*, 69 99–100, 1948

21 Kilham, L Mumps Virus in Human Milk and in Milk of Infected Monkey *J A M A*, 146 1231–1232, 1951

22 Weller, T H, and Craig, J M The Isolation of Mumps Virus at Autopsy *Am J Path* 25 1105–1115, 1949

23 Habel K Cultivation of Mumps Virus in the Developing Chick Embryo and Its Application to Studies of Immunity to Mumps in Man *Pub Health Rep*, 60 201–212 1945

24 Levens J H, and Enders, J F The Hemoagglutinative Properties of Amniotic Fluid from Embryonated Eggs Infected with Mumps Virus *Science*, 102 117–120, 1945

25 Kilham, L, and Murphy, H W Propagation of Mumps Virus in Suckling Mice and in Mouse Embryo Tissue Cultures *Proc Soc Exper Biol and Med*, 80 495–498, 1952

26 Kilham, L, and Overman J R Natural Pathogenicity of Mumps Virus for Suckling Hamsters on Intracerebral Inoculation *J Immunol*, 70 147–151, 1953

27 Beveridge, W. I B., and Burnet F M The Cultivation of Viruses and Rickettsiae in the Chick Embryo *Medical Research Council, Special Report Series No 256*, 1946

sence of salivary gland involvement, the hemagglutination inhibition test is probably the most practical and easily performed The same information may be gained from a complement fixation test with either the crude or the soluble or virus antigens, but these tests are not so easy to perform. For final diagnosis paired serum specimens are required both in the hemagglutination inhibition test and in the complement fixation test. Therefore, the results cannot be obtained much before the end of the 1st and frequently not before the end of the 2d week. By using the S and V antigens on a single specimen of serum the complement fixation test may provide an early presumptive diagnosis in possibly two-thirds of all cases of meningoencephalitis It should be borne in mind that occasionally one type of test may fail to yield unequivocal results. Under such circumstances it is advisable to employ an alternative procedure.

For measuring immunity to mumps whether it be that resulting from natural infection or the application of specific immunization procedures, the virus neutralization test, though it is a cumbersome technic, affords the most significant and consistent results In evaluating natural immunity the skin test is a more practical substitute for the neutralization test. Likewise, a positive complement fixation test with virus antigen furnishes a good criterion of immunity due to past infection. A negative complement fixation test, however, does not signify susceptibility.

Each of the diagnostic tests in mumps has been separately discussed and evaluated Comparisons of the results of these tests in large numbers of individuals have revealed a lack of close quantitative correlation. In fact, where evidence of past immunity is being sought, some tests will be negative and others will be positive The absence of complete agreement between the results obtained by the different procedures may in part depend upon the existence of different antibodies that react with different components of the virus Indeed, there is suggestive evidence[32,67] that the different serologic technics measure different antibodies and that dermal sensitivity is not directly related to serum antibody levels [14]

IX. REFERENCES

1 Rolleston, J D Some Aspects of Mumps, A Review of Recent Literature. *Practitioner*, 128 31–39, 1932.

2 Wesselhoeft, C Mumps Its Glandular and Neurologic Manifestations In *Virus and Rickettsial Diseases* Cambridge, Mass Harvard University Press, 1940, pp 309–348

3 Wesselhoeft, C Mumps *Oxford Medicine*, vol V, part II, 1941, pp 489–497 (36)

4 Van Rooyen, C. E, and Rhodes, A J *Virus Diseases of Man* London Oxford University Press, 1940, pp 234–249

5 Gordon, J E, and Kilham, L Ten Years in the Epidemiology of Mumps *Am J M Sc*, 218 338–359, 1949

6 Leymaster, G R, and Ward, T G Direct Isolation of Mumps Virus in Chick Embryos *Proc. Soc Exper Biol and Med*, 65 346–348, 1947

7 Henle, G, Henle, W, Wendell, K K, and Rosenberg, P Isolation of Mumps Virus from Human Beings with Induced Apparent or Inapparent Infections *J Exper Med*, 88 223–232, 1948

8 Kilham, L Mumps Meningoencephalitis with and without Parotitis *Am J Dis Child*, 78 324–333, 1949

9 Kane, L W, and Enders, J F Immunity in Mumps III The Complement Fixation Test as an Aid in the Diagnosis of Mumps Meningoencephalitis *J Exper Med*, 81 137–150, 1945

10 Kravis, L P Sigel, M M, and Henle, G Mumps Meningoencephalitis with Special Reference to the Use of the Complement-Fixation Test in Diagnosis *Pediatrics*, 8 204–215, 1951

11 Applebaum, I L Serum Amylase in Mumps *Ann Int Med*, 21 35–43, 1944

12 Candel, S, and Wheelock, M C Serum Amylase and Serum Lipase in Mumps *Ann Int Med*, 25 88–96, 1946

13 Wolman, I J, Evans, B, Lasker, S, and Jaegge, K Amylase Levels during Mumps, Findings in Blood and Saliva *Am J M Sc* 213 477–481, 1947

14 Enders, J F, Cohen, S, and Kane, L W Immunity in Mumps II The Development of Complement-Fixing Antibody and Dermal Hypersensitivity in Human Beings Following Mumps *J Exper Med*, 81 119–135, 1945

15 Habel, K Vaccination of Human Beings against Mumps Vaccine Administered at the Start of an Epidemic I Incidence and Severity of Mumps in Vaccinated and Control Groups *Am J Hyg* 54 295–311, 1951

16 Enders, J F Unpublished data

17 Enders, J F, Kane L W, Cohen S, and Levens, J H Immunity in Mumps I Experiments with Monkeys (*Macacus mulatta*) The Development of Complement-Fixing Antibody Following Infection and Experiments on Immunization by Means of Inactivated Virus and Convalescent Human Serum *J Exper Med*, 81 93–117, 1945

18 Henle, G, Henle, W, and Harris, S The Serological Differentiation of Mumps Complement-Fixation Antigens *Proc Soc Exper Biol and Med*, 64 290–295, 1947

19 Henle, G, and McDougall, C L Mumps Meningo-Encephalitis Isolation in Chick Embryos of Virus from Spinal Fluid of a Patient *Proc Soc Exper Biol and Med*, 66 209–211, 1947

20 Kilham, L Isolation of Mumps Virus from the Blood of a Patient *Proc Soc Exper Biol and Med*, 69 99–100, 1948

21 Kilham, L Mumps Virus in Human Milk and in Milk of Infected Monkey *J A M A*, 146 1231–1232, 1951

22 Weller, T H, and Craig, J M The Isolation of Mumps Virus at Autopsy *Am J Path*, 25 1105–1115, 1949

23 Habel, K Cultivation of Mumps Virus in the Developing Chick Embryo and Its Application to Studies of Immunity to Mumps in Man *Pub Health Rep*, 60 201–212, 1945

24 Levens J H, and Enders, J F The Hemoagglutinative Properties of Amniotic Fluid from Embryonated Eggs Infected with Mumps Virus *Science* 102 117–120, 1945

25 Kilham L, and Murphy, H W Propagation of Mumps Virus in Suckling Mice and in Mouse Embryo Tissue Cultures *Proc Soc Exper Biol and Med*, 80 495–498, 1952.

26 Kilham [illegible]

27 Be

28 Weil, M. L, Beard, D, Sharp, D G, and Beard, J W. Purification, pH Stability and Culture of the Mumps Virus. *J Immunol*, 60·561–582, 1948.
29 Needham, J *Chemical Embryology* Vol II New York McMillan Co, Cambridge, England The University Press 1931, pp 1100–1101.
30 Beveridge, W I B, Lind, P E, and Anderson, S. G Mumps I Isolation and Cultivation of the Virus in the Chick Embryo. *Australian J Exper Biol and M Sc*, 24 15–19, 1946
31 Johnson, C D, and Goodpasture, E W An Investigation of the Etiology of Mumps *J Exper Med*, 59 1–19, 1934
32 Enders, J F, Levens, J H, Stokes, J, Jr, Maris, E P, and Berenberg, W. Attenuation of Virulence with Retention of Antigenicity of Mumps Virus after Passage in the Embryonated Egg *J Immunol*, 54 283–291, 1946
33 Johnson, C D, and Goodpasture, E W Experimental Immunity to the Virus of Mumps in Monkeys *Am J Hyg*, 23 329–339, 1936
33a Henle, G, and Deinhardt, F Propagation and Primary Isolation of Mumps Virus in Tissue Culture *Proc Soc Exper Biol and Med*, 89 556–560, 1955
34 Wollstein, M An Experimental Study of Parotitis (Mumps). *J Exper. Med*, 23 353–375, plus 3 plates, 1916.
35 Bolin, V S, Anderson, J A, and Leymaster, G. R Experimental Intraocular Infection of Guinea Pig with Mumps Virus *Proc. Soc Exper Biol and Med*, 75.166–170, 1950
36 Bolin, V S, and Leymaster, G R The Use of Guinea Pig Eye in Study of Intraocular Infections Produced by Mumps Virus *Proc Soc Exper Biol and Med*, 79 7–12, 1952
37 Burr, M M, and Nagler, F P Mumps Infectivity Studies in Hamsters *Proc Soc Exper Biol and Med*, 83 714–717, 1953.
38 Kilham, L, and Murphy, H W Propagation of Mumps Virus in Suckling Mice and in Mouse Embryo Tissue Cultures *Proc Soc Exper Biol and Med*, 80 495–498, 1952
38a Kilham, L, and Overman, J R Natural Pathogenicity of Mumps Virus for Suckling Hamsters on Intracerebral Inoculation *J. Immunol*, 70 147–151, 1953.
39 Enders, J F Mumps Chapter 28 in Rivers, T M, *Viral and Rickettsial Infections of Man* 2d ed Philadelphia, J B Lippincott Company, 1952, pp 513–521
40 Kilham, L Personal communication
41 Maris, E P, Enders, J F, Stokes, J, Jr, and Kane, L W. Immunity in Mumps IV The Correlation of the Presence of Complement-Fixing Antibody and Resistance to Mumps in Human Beings. *J Exper Med*, 84 323–339, 1946.
42 Henle, G, Henle, W, Burgoon, J. S, Bashe, W. J, Jr, and Stokes, J, Jr, Studies on the Prevention of Mumps I The Determination of Susceptibility *J Immunol*, 66 535–549, 1951
43. Stokes, J, Jr, Enders, J F, Maris, E P, and Kane, L W. Immunity in Mumps VI Experiments on the Vaccination of Human Beings with Formolized Mumps Virus *J Exper Med*, 84 407–428, 1946
44 Henle, G, Stokes, J, Jr, Burgoon, J S, Bashe, W. J., Jr, Burgoon, C F, and Henle, W Studies on the Prevention of Mumps IV The Effect of Oral Spraying of Attenuated Active Virus *J Immunol*, 66 579–594, 1951
45 Habel, K Preparation of Mumps Vaccines and Immunization of Monkeys against Experimental Mumps Infection *Pub Health Rep*, 61 1655–1664, 1946
46 Oldfelt, V Complement Fixation Tests for Diagnosis of Mumps Applied to Typical and Suspect Cases of Mumps Meningo-Encephalitis *Acta Med Scandinav*, 133 98–112, 1949
47 Enders, J. F, and Levens, J H Unpublished data
48 Henle, G, Harris, S, and Henle, W The Reactivity of Various Human Sera with Mumps Complement Fixation Antigens *J Exper Med*, 88 133–147, 1948.

48a Henle, W, and Henle, G Interference between Inactive and Active Viruses of Influenza II Factors Influencing the Phenomenon *Am J M Sc*, 207 717–723, 1944

49 Mayer, M M, Osler, A G, Bier, O G, and Heidelberger, M Quantitative Studies of Complement Fixation I A Method *J Immunol*, 59 195–206, 1948

50 Bukantz, S C, Rein, C R, and Kent, J F Studies in Complement Fixation II Preservation of Sheep's Blood in Citrate Dextrose Mixtures (Modified Alsever's Solution) for Use in the Complement Fixation Reaction *J Lab Clin Med*, 31 394–399, 1946

51 Henle, G, Burgoon, J S Bashe, W J, Jr, Burgoon, C F, Stokes, J, Jr, and Henle, W Studies on the Prevention of Mumps II The Effect of Skin Testing upon Antibody Level and Resistance *J Immunol*, 66 551–560, 1951

52 Kilham, L, Jungherr, E, and Luginbuhl, R E Antihemagglutinating and Neutralizing Factors against Newcastle Disease Virus (NDV) Occurring in Sera of Patients Convalescent from Mumps *J Immunol*, 63 37–49, 1949

53 Jordan, W S, Jr, and Feller, A E The Relationship of Complement-Fixing and Antihemagglutinating Factors against the Viruses of Mumps and Newcastle Disease *J Lab and Clin Med*, 36 369–377, 1950

54 Wenner, H A, Jenson, M H and Monley, A A Study of Infections Caused by Mumps and Newcastle Disease Viruses I Specific and Non-specific Serologic Relations *J Immunol*, 68 343–356 1952

55 Wenner, H A, Monley, A and Jenson, M H A Study of Infections Caused by Mumps and Newcastle Disease Viruses II Some Properties of Specific and Nonspecific Serum Hemagglutination Inhibitor Components *J Immunol*, 68 357–368, 1952

56 Hirst, G K Quantitative Determination of Influenza Virus and Antibodies by Means of Red Cell Agglutination *J Exper Med*, 75 49–64, 1942

57 Nagler, F P O Application of Hirst's Phenomenon to Titration of Vaccinia Virus and Vaccinia Immune Serum *M J Australia*, 1 281–283, 1942

58 Burnet, F M The Affinity of Newcastle Disease Virus to the Influenza Virus Group *Australian J Exper Biol and M Sc*, 20 81–88, 1942

59 Burnet, F M, and Boake, W C The Relationship between the Virus of Infectious Ectromelia of Mice and Vaccinia Virus *J Immunol*, 53 1–13, 1946

60 Florman, A L "False Positive" Hemagglutination by Allantoic Fluids of Embryonated Eggs Inoculated with Unfiltered Throat Washings *J Bact*, 52 307–310, 1946

61 Commission on Acute Respiratory Diseases Hemagglutination by Amniotic Fluid from Normal Embryonated Hen's Eggs *Proc Soc Exper Biol and Med*, 62 119–123, 1946

62 Robbins, F C, Kilham, L, Levens, J H, and Enders, J F An Evaluation of the Test for Antihemagglutinin in the Diagnosis of Infections by the Mumps Virus *J Immunol*, 61 235–242 1949

63 Feller, A E and Jordan, W S, Jr Serologic Studies of Mumps Employing Complement Fixation and Agglutination-Inhibition *J Lab and Clin Med*, 36 360–368, 1950

64 Aikawa, J K, and Meiklejohn, G The Serologic Diagnosis of Mumps A Comparative Study of Three Methods *J Immunol*, 62 261–269, 1949

65 Henle, G, Bashe, W J, Jr, Burgoon, J S, Burgoon C F, Hunt, G. R., Jr, and Henle, W Studies on the Prevention of Mumps III The Effect of Subcutaneous Injection of Inactivated Mumps Virus Vaccines *J Immunol*, 66 561–577, 1951

66 Salk, J E A Simplified Procedure for Titrating Hemagglutinating Capacity of Influenza Virus and the Corresponding Antibody *J Immunol*, 49 87–98, 1944

67 Gotlieb, T, Bashe, W J Jr, Henle, G, and Henle, W Studies on the Prevention of Mumps V The Development of a Neutralization Test and Its Application to Convalescent Sera *J Immunol*, 71 66–75, 1953

68 Leymaster, G R, and Ward, T G. The Effect of ... Test of M...

69 Habel, K ... 39 785

70. Kilham, L, Murphy, H W, and Overman, J. R. Performance of Mumps Neutralization Tests in Suckling Mice J Immunol, 71 183–186, 1953

71. Bashe, W J, Jr, Gotlieb, T, Henle, G., and Henle, W Studies on the Prevention of Mumps VI The Relationship of Neutralizing Antibodies to the Determination of Susceptibility and to the Evaluation of Immunization Procedures J. Immunol, 71 76–85, 1953

72 Ray, B G, and Swain, R. H A Serum Antibodies in Mumps Brit J. Exper. Path., 34 501–511, 1953.

73 Enders, J F Observations on Immunity in Mumps Ann Int Med, 18:1015–1019, 1943

74 Enders, J F, Kane, L W, Maris, E P, and Stokes, J, Jr Immunity in Mumps V. The Correlation of the Presence of Dermal Hypersensitivity and Resistance to Mumps J Exper Med, 84 341–364, 1946.

75 Muntz, H H, Powell, H M, and Culbertson, C. G I Mumps Vaccine Studies on Human Volunteers J Lab and Clin Med, 34 199–208, 1949

76 Florman, A L, Rischer, A E, and Moloshok, R E Evaluation of Mumps Skin Test Pediatrics, 5 469–473, 1950

HERPES SIMPLEX

T. F McNair Scott, M D.

Research Professor of Pediatrics, University of Pennsylvania, and Director of Research, The Children's Hospital of Philadelphia

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I INTRODUCTION

SINCE 1919[1,2] it has been recognized that the virus of herpes simplex (*Herpesvirus hominis*) could be isolated from "fever blisters" or "cold sores" (herpes simplex, herpes febrilis), and the frequency of their occurrence makes it one of the commonest virus agents associated with human disease. Within the last 15 years, however, it has been realized that this virus plays a much wider role in human pathology than as the causative agent of the clinical entity of herpes simplex.

The host responds to primary infection with this virus, as to any other infection, by the development of antibodies In addition clinical disease may be manifest but more often, probably 80 to 90 per cent of the time, the primary infection is subclinical When clinical infection occurs the illness may be severe and occasionally fatal, manifesting both local and systemic signs and symptoms After a primary infection, either clinical or subclinical, the host becomes a carrier of a latent virus and is subject to the common recurrent form of the disease in which local but not systemic symptoms occur It will be useful to summarize the varied clinical forms under which the disease may appear.

A Diseases Caused by the Virus [3]

1 *Diseases of the skin*

a Recurrent herpes simplex This is as an eruption of sharply defined, closely grouped, thin-walled vesicles on an erythematous base which may occur on any part of the skin but has a particular predilection for mucocutaneous junctions and frequently recurs in the same place

b Eczema herpeticum (Kaposi's varicelliform eruption) This should be suspected if a patient with eczema becomes suddenly febrile and develops crops of vesicles over the eczematous areas These quickly pustulate and scab

c Traumatic herpes The herpes virus may become inoculated into any portion of traumatized skin, such as a graze or burn and may lead to a primary infection with a vesicular eruption, enlarged regional lymph nodes, and some fever Lesions may recur in the area of trauma

2 *Diseases of the mucous membranes*

a Acute infectious gingivostomatitis This is probably the commonest clinical form of the primary infection and the commonest form of stomatitis between the ages of 1 and 3 years It may occur at any age, even in adults Scattered, painful, collapsed vesicles covered with a gray-yellowish membrane appear in the mouth, accompanied by high fever and irritability Once healed, herpetic stomatitis seldom, if ever, recurs, and the bulk of the evidence is against recurrent aphthous ulcers being herpetic in origin

b Keratoconjunctivitis, primary or recurrent, may show itself as a conjunctivitis or more commonly as a keratoconjunctivitis It should be suspected if there is (1) little or no purulent exudate, especially if the exudate is bacteriologically sterile, (2) an enlarged preauricular node, (3) a dendritic or other type of corneal ulceration; and (4) herpetic vesicles on the eyelids

c Vulvovaginitis, primary or recurrent In primary vulvovaginitis, scattered collapsed vesicles, covered with a gray-yellowish membrane (see 2a above) occur over the labiae and are accompanied by enlarged, tender, inguinal nodes and some fever In recurrent vulvovaginitis the lesions appear much the same, but they tend to be more grouped, and there is less adenopathy and fever

d Progenitalis is common in the recurrent form in which grouped vesicles occur on the glans and on the shaft of the penis

3 *Diseases of the central nervous system*

Meningoencephalitis or encephalitis may result from the primary infection with herpes simplex virus These cases have no determining clinical characteristics by which they can be distinguished from central nervous system involvement caused by other agents The encephalitic type is acute and may be rapidly fatal, on the other hand, many cases fall into the category of acute aseptic meningitis As routine diagnostic tests for herpes are applied to this group, an increasing incidence of this infection is being uncovered

4 *Systemic diseases*

a Occasionally the primary infection is manifest mainly as a generalized systemic illness resembling grippe or infectious mononucleosis. When such are accompanied by a generalized vesicular rash, they may be mistaken for smallpox.

b A fulminating visceral disease may occur in newborn infants,[4] particularly if they are premature This is characterized by vesicular lesions of the conjunctivae and skin, although these may be absent or very inconspicuous, associated with fever or hypothermia, jaundice, lethargy, dyspnea, pneumonitis, and enlarged liver, followed by circulatory collapse and death

From some patients with clinical manifestations of primary infection with the virus—for example, acute infectious gingivostomatitis—it is possible to get a history of contact with someone suffering from herpes, usually herpes labialis, to provide a source of the virus In others, no obviously infected contact can be found In such cases, it must be assumed that an asymptomatic contact is carrying the virus, probably in the saliva It is known that virus can be isolated from the saliva of apparently normal persons,[5] but the factors that influence the susceptibility to clinical illness of a recipient of such infected saliva, by droplets or otherwise, are imperfectly understood Certainly upper respiratory infection and trauma are commonly associated with the onset of an acute gingivostomatitis, whereas trauma of the skin in the form of eczema or mechanical abrasions paves the way for an implanted herpetic infection

B. The Virus and Its Physical and Pathogenic Characteristics

The virus of herpes simplex has the following characteristics

1 *Size* 90 to 213 mμ[6,10,15,16,17] in diameter.

2. *Filtration.* When suspended in broth it can pass a Berkefeld V filter candle[7] It can also pass through collodion membranes of porosity greater, but not less, than 200 mμ From this, the size was calculated as 100 to 150 mμ[6]

3. *Centrifugation.* It can be sedimented at 5,000 r.p m in 2 to 2½ hours[8] Centrifugation at 11,000 r p m for 1 hour in an angle high-speed centrifuge (Sorvall) at 4° C. sedimented 96 to 98 per cent of the virus from egg amniotic fluids containing from 10 to 30,000,000 particles/ml.[9]

An average sedimentation constant of 1,178 S (range 1,000 S to 1,200 S) has been reported[10] for highly purified, egg-adapted virus

4. *Electrical reaction.* By a simple filter-paper method the isoelectric point was found to be between pH 7 2 and 7 6, the virus being negatively charged between pH 7 6 and 8.0 and positively charged between pH 6 6 and 7 2[11], whereas by electrophoretic methods it was found that the particles were negatively charged from pH 6 8 to 7 8[12]

5 *Visibility.*

a Light microscope In highly purified preparations elementary bodylike objects can be demonstrated by dark field illumination or by staining with Giemsa[13] or Victoria blue.[14]

b Electron microscope Extensive studies of sections through infected chorioallantoic membranes by Morgan *et al.*[17] have revealed the steps in development of the virus particles in the cell These range from small, dense primary bodies, each 30 to 40 mμ in diameter surrounded by a single membrane and situated in the nucleus, to larger, less dense particles each 120 to 130 mμ in diameter These are situated in the cytoplasm and are surrounded by a double membrane Other studies[10,15,16 18] of free virus have described the particles as spherical and inhomogeneous, with either a dense central portion or a central depression which gave a doughnut-like shape to the particle. In these studies the average diameter has varied in size from 90 to 213 mμ The lower limit is more compatible with the diameter as calculated from the sedimentation constant of purified preparations [10]

6 *Susceptible hosts* are man, rabbit, mouse, guinea pig, cotton rat, hamster, the embryonated hen's egg, and tissue cultures of HeLa cells[10] or L strain of mouse fibroblasts [19a]

7. *Histologic characteristics* There are two histologic features that are characteristic of, but not peculiar to, herpes simplex virus infections

a The presence of intranuclear inclusion bodies which are found in all types of infected tissue The morphologic and tinctorial properties of these bodies vary, probably with age [20,21] Early they appear as a homogeneous material which fills the center of the nucleus up to the marginated basichromatin and stains bluish with hematoxylin and eosin Later the inclusion appears to shrink away from the basichromatin and finally appears as a pink staining object surrounded by a halo

which separates it from the marginated chromatin (Type A inclusion of Cowdry).[22] There is usually severe tissue reaction in the neighborhood of Type A inclusions.

b The presence of "ballooning cells," which are found in epithelial tissue only.

A more detailed description of the pathologic picture seen in the various tissues infected by the virus is given later in connection with the technics involved in the proper diagnosis of this infection.

II METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

A. Special Precautions

The virus is not dangerous for most of those working with it since the majority of adults have neutralizing antibodies against the virus and even for those without, there is only a minimum chance of an infection being clinical in character Susceptibles with atopic eczema should not work with the virus. As a rule, no special precautions need be observed

B. Sources of Material and Collection of Specimens

The shortest possible time should be allowed to elapse between the collection and the inoculation of the specimen. If delay is inevitable the specimen should be kept frozen

Since the virus causes a variety of clinical symptoms, several types of material will be studied for the presence of virus, each of which must be handled appropriately Many specimens will need treatment for the removal of bacterial contaminants before inoculation. The following technic has been found useful in most cases·

Allow the material to be inoculated to stand for one-half hour at room temperature, with one-tenth its volume of the following mixture

Penicillin	..5,000 units/ml
Streptomycin	..1,000 μg/ml

In order to avoid fungus contamination it is wise to add Mycostatin (Squibb Nystatin) 10,000 units/ml to the material being inoculated 200,000 units (1 ampule)

sufficient of the diluted stock to the inoculum to equal 1/10 total volume

1. *Swabs from ulcers of mouth, eye, or genitalia.* The swab should

preferably be moistened with a suitable solution* but can be used dry. It should be firmly applied to the ulcer and then placed in a sterile tube containing a few drops, to 0.5 ml, of the solution chosen Physiologic saline solution should be used only if no other solution is available—see under Section I. If not to be inoculated at once, the tube should be quick-frozen and placed in the freezing compartment of a refrigerator, or in a deep-freeze unit, at –15° C to –20° C If facilities for freezing are not available, an equal amount of sterile neutral glycerol should be added to the solution, and the tube should be kept in a refrigerator until used.

2. *Vesicle fluid* The fluid should be collected from a fresh vesicle. This is done most efficiently by using 1 or 2 fine glass capillary tubes If experimental animals are immediately available, the contents of the tubes can be expressed onto the prepared site by means of a small rubber bulb such as is used with smallpox vaccine vials It should be noted that vesicle fluid clots in the capillaries on standing. If the contents cannot be expressed by blowing, the glass tubes can be pulverized in a little diluent and, after clearing by centrifugation, the supernatant fluid can be used for inoculation Also, fluid can be withdrawn by means of a fine needle (26- or 27-gauge) and syringe. If sufficient material is not thus obtainable the vesicle can be washed out with gelatine-saline and the washings inoculated If the animals are not immediately available, the material must be preserved by freezing, either in the capillaries sealed at the ends or in a small test tube.

3 *Saliva* This should be collected in a glass receptacle and used at once or transferred to a test tube and kept frozen

4 *Cerebrospinal fluid* This should be collected with the usual aseptic precautions and either used immediately or preserved frozen

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* Suitable solutions



bacto-gelatin (Difco) (0 5%) and heat to dissolve

(*b*) Nutrient broth This may cause excessive reaction in mouse brain or on the chorioallantoic membrane of eggs if used full strength

(*c*) Physiologic saline + 10% normal horse or rabbit serum

(*d*) Buffered saline + 0 1% yolk[204]

5. *Brain or spinal cord.* Portions of the brain or spinal cord should be taken at autopsy, using all possible precautions to minimize bacterial contamination. Some portions should be placed in sterile neutral 50 per cent glycerol and saline, and others should be preserved by freezing It has been shown[24] that encephalitogenic strains of the virus are often more easily demonstrable after the material has been stored in glycerol

6. *Blood.* This may be tested as follows After aseptic removal, it is allowed to clot, and the serum separated. The clot can be preserved in 50 per cent glycerol or ground with sterile alundum (90 mesh) in a small quantity of suitable diluent * After centrifugation at approximately 2,000 r.p m for 10 minutes to remove the coarse debris, the supernatant is either inoculated immediately or kept frozen

If specimens must be shipped, they should be preserved in 50 per cent neutral glycerol, in rubber-stoppered tubes, and sent by the quickest postal or air express routes.

C Isolation Procedures

There are four readily available laboratory hosts that are about equally well suited to the primary isolation of the herpes virus. They are in order of preference, the embryonated hen's egg, HeLa cell tissue culture, the suckling mouse, and the rabbit After primary isolation the virus can be adapted to the guinea pig, hamster, and cotton rat and can sometimes be isolated in these hosts.

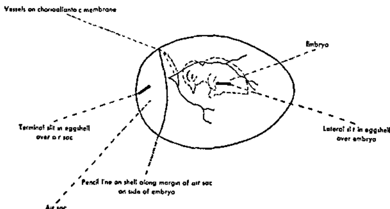
1 *Embryonated eggs.* The chorioallantoic membrane has been shown by a number of workers to be highly susceptible to this virus. Extensive discussions of technics used for growing viruses in the embryonated egg are given by Beveridge and Burnet[25] and Buddingh[25a] The following technics have been used very satisfactorily in this laboratory·

a. All bacterially contaminated or potentially contaminated material should be treated with the antibiotic mixture given above.

b. Twelve- to 13-day-old embryonated eggs, in which the chorioallantoic membrane is well developed, should be used and prepared as follows (1) candle the egg and pencil in the margin of the air sac on the side of the shell corresponding to the embryo (see sketch); (2) cleanse the shell over the embryo with 70 per cent alcohol; (3) with a grinder and a carborundum disk, 1 inch in diameter, carefully grind a

* See footnote on page 319

broad slit, approximately ⅛ to ¼ inch long in the long axis of the shell, down to but not through the shell membrane Wetting the surrounds of the slit with an alcohol sponge will blacken the membrane when the shell has been perforated Perforate the shell and shell membrane over the air sac with a drill (4) With the egg lying horizontally in a suitable holder,* resterilize with alcohol and place a drop of saline over the lateral slit with a syringe of sterile saline and a 22-gauge needle,



then, with a gentle rocking motion, insert the needle, bevel up and almost parallel to the shell, through the saline in such a way as to pierce the shell membrane Withdraw the needle When the shell membrane has been pierced the drop of saline will slowly seep into the egg between the shell membrane and the chorioallantoic membrane, (5) with the egg over the candling box apply suction to the terminal hole over the air sac, watching a "false" air sac form by the entrance of the air through the slit placed over the embryo. A disappearance from view of the clearly marked blood vessels indicates the entrance of air between the shell membrane and the chorioallantoic membrane If the membrane does not drop on the first trial another drop of saline will generally lead to dropping of the membrane In order to minimize trauma to the chorioallantoic membrane the least amount of suction should be used compatible with forming the sac (6) Take up 0 05 ml. of the material to be injected in

* A convenient holder is a fiberboard egg tray which holds 30 eggs These come in nests of 140 trays and bear the name of "News Filler Flats" They can be procured from Keyes Fibre Company, Waterville, Maine

a ½ or ¼ ml. tuberculin syringe and, using a 27-gauge ¼-inch needle, inject it into the false air sac over the embryo. (6a) An alternative method used by Beveridge and Burnet[25] consists in using the material to be inoculated to form the air sac. A rather broader slit is drilled through the shell down to the shell membrane. The surrounds of the slit are rimmed with melted paraffin and the shell membrane clarified with the smallest possible drop of mineral oil 0.05 ml. of the material to be inoculated is placed over the slit. The shell membrane is split by a combined pressure and dragging movement of a blunted glass needle through the drop and false air sac formed by suction over the air sac as above This procedure in skillful hands minimizes trauma to the allantoic membrane (7) The slits in the shell do not need covering. (8) Incubate eggs at 36° C. for 36 to 48 hours on their sides with false air sac uppermost. (9) Remove from incubator and candle again If the normal air sac has re-formed, as it does occasionally, leaving the chorioallantoic membrane up against the shell membrane, suck the air out of the normal sac to re-form the false one. (10) Open the eggshell with scissors, outward from the slit, and, under direct vision, push down the chorioallantoic membrane from off the shell, taking off the shell so freed. Allow the escape of some fluids and yolk. This allows the whole of the "dropped" area of the chorioallantoic membrane to be completely exposed. Cut around this with scissors (11) Remove the excised part of the membrane to a petri dish containing 1 per cent formalin. The outer surface of the bottom of the dishes used for this purpose should be blackened with enamel paint (12) Flatten out the excised membrane and examine under a good light with a 2X lens (13) If virus is present, the membrane will either show a marked generalized thickening over the inoculated area or a mass of confluent grayish white pocks, or numerous small discrete pocks varying in size from 0 5 to 2.0 mm , depending on the amount of virus present.

Beveridge and Burnet[25] suggest the following general criteria for differentiating specific from nonspecific lesions seen on egg membranes The specific lesion tends to have (1) a circular shape, (2) a more opaque central part, which may become necrotic, and (3) a surrounding haze due to mesodermal infiltration with inflammatory cells This description fits the pocks of vaccinia virus perfectly. In herpes, however, these characteristics are modified in that the pocks tend to be oval or actually have a tail, and neither necrosis nor the mesodermal haze is prominent The pocks give the impression of being very superficial and can in fact be scraped off the membrane On primary isolation they may be very small and must be searched for with magnification in a good light

For passage the chorioallantoic membrane is removed according to the above technic, except that the usual diluent is used in place of formalin, and ground with alundum and gelatin saline After centrifugation at 1,500 to 2,000 r p m to remove coarse particles, the supernatant fluid is inoculated onto the chorioallantoic membrane of 12-day eggs as described above It may take several passages before typical pocks develop, and any membrane which shows suggestive pocks or only edema, even in the presence of obvious nonspecific irritation (thickening and irregular opacities along the blood vessels) should be passed in series onto other eggs at least three times

2 *Tissue culture cells* HeLa cells are grown in tissue culture according to standard technics Material suspected of containing herpes virus is prepared as for inoculation into eggs and inoculated in 0 1 ml amounts into each of 2 tubes containing sheets of cells growing in maintenance medium The tubes are incubated at 37° C. in stationary racks The presence of virus is recognized by the appearance of a characteristic cytopathogenic effect which may appear as early as 2d or 3d day if the virus content is high Usually there appears a pock with piling up of cells or there is a rounding up of cells with a consequent break in the sheet Intranuclear inclusions can be demonstrated in the affected cells These foci are scattered at first but later may involve the whole tube

3. *Suckling mice* These have been shown to be highly susceptible[26] to infection with the herpes virus 0 05 ml of suspected material, sterilized bacteriologically as above, is injected intraperitoneally into 1-day-old mice Evidence of infection appears on the 3d day after inoculation, deaths before the 36th hour are nonspecific. On the 3d day hyperactivity and undernutrition are manifest followed by cyanosis, abdominal distention, and death Death is delayed when very small quantities of virus are inoculated or slightly older mice used, being preceded by an ascending spastic paralysis This paralysis inevitably precedes death

4 *Rabbits*. There are two methods of inoculation

a Corneal route. This has long been known to be suitable for isolation of the herpes virus[1,2] from all superficial or bacterially contaminated tissues without preliminary antibiotic treatment. The technic is as follows:

Under ether or nembutal anesthesia, protrude one eyeball of the rabbit by firm pressure against the lower lid with the butt end of a pair of forceps Steadying the eye by this means, scarify the cornea thorough-

a ½ or ¼ ml tuberculin syringe and, using a 27-gauge ¼-inch needle, inject it into the false air sac over the embryo. (6a) An alternative method used by Beveridge and Burnet[25] consists in using the material to be inoculated to form the air sac. A rather broader slit is drilled through the shell down to the shell membrane. The surrounds of the slit are rimmed with melted paraffin and the shell membrane clarified with the smallest possible drop of mineral oil 0.05 ml. of the material to be inoculated is placed over the slit The shell membrane is split by a combined pressure and dragging movement of a blunted glass needle through the drop and false air sac formed by suction over the air sac as above. This procedure in skillful hands minimizes trauma to the allantoic membrane (7) The slits in the shell do not need covering (8) Incubate eggs at 36° C for 36 to 48 hours on their sides with false air sac uppermost (9) Remove from incubator and candle again If the normal air sac has re-formed, as it does occasionally, leaving the chorioallantoic membrane up against the shell membrane, suck the air out of the normal sac to re-form the false one. (10) Open the eggshell with scissors, outward from the slit, and, under direct vision, push down the chorioallantoic membrane from off the shell, taking off the shell so freed Allow the escape of some fluids and yolk. This allows the whole of the "dropped" area of the chorioallantoic membrane to be completely exposed. Cut around this with scissors (11) Remove the excised part of the membrane to a petri dish containing 1 per cent formalin. The outer surface of the bottom of the dishes used for this purpose should be blackened with enamel paint. (12) Flatten out the excised membrane and examine under a good light with a 2X lens. (13) If virus is present, the membrane will either show a marked generalized thickening over the inoculated area or a mass of confluent grayish white pocks, or numerous small discrete pocks varying in size from 0 5 to 2 0 mm , depending on the amount of virus present.

Beveridge and Burnet[25] suggest the following general criteria for differentiating specific from nonspecific lesions seen on egg membranes The specific lesion tends to have (1) a circular shape, (2) a more opaque central part, which may become necrotic, and (3) a surrounding haze due to mesodermal infiltration with inflam- [illegible] perfectly In herpes, [illegible] oval or [illegible] prominent

The pocks give the impression of being very superficial and can in fact be scraped off the membrane On primary isolation they may be very small and must be searched for with magnification in a good light

diluent,* centrifuged to remove coarse particles, and the supernatant inoculated intracerebrally into another rabbit or other passage animal

5. *Other animals.* When the virus is passaged to hosts other than the four just described, the following disease patterns may be seen:

a Adult mice After intracerebral inoculation, the animals on primary isolation may not show symptoms for 5 to 7 days but, in a well-adapted strain, they may show symptoms as early as 24 hours or, more usually, 2 to 4 days after inoculation Usually, the first significant sign is marked jumpiness and hyperactivity on stimulation A knock on the cage sets them leaping, convulsions occur when they are spun by the tail, then the fur becomes roughened, the gait spastic, and activity lessens Death often occurs after a tetanic convulsion, with the front and hind legs extended backwards, a position similar to that seen in infection with lymphocytic choriomeningitis They are often found dead, however, without showing any typical position After intraperitoneal inoculation, the incubation period is longer, and the mortality is usually lower than after intracerebral inoculation The animals appear sluggish, with roughened fur. Typical convulsive seizures are not so common as after intracerebral inoculation, the mice dying rather unexpectedly Virus is present in the brain after intraperitoneal inoculation [29]

b Guinea pigs Apart from keratoconjunctivitis after corneal inoculation, which does not differ essentially from that of the rabbit, dermal strains of the virus can produce intracutaneous and footpad lesions The inoculated footpads become inflamed and swollen on the second day, vesicles then develop along the needle tract and coalesce to form a single large vesicle This heals slowly with considerable scarring More satisfactory is the inoculation of virus onto scarified and epilated skin Vesicles appear by the second day and slowly heal [30] After intracerebral inoculation, the animals may develop fever and occasionally convulsions [29]

c. Hamsters [29] After intracerebral inoculation, these animals show chiefly hyperactivity, then sluggishness, muscular inco-ordination, tremors, and, in some instances, paralysis and death

d Cotton rats This species has been reported as being very susceptible to the virus by the intranasal route as well as by the intracerebral route.[31]

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* See footnote on page 319

ly with vertical and horizontal strokes of a von Graefe knife or other suitable instrument, taking care that the incisions actually go through the epithelial layer. Turn the rabbit over and repeat the procedure on the other cornea. Take the infected swab and, with the eyes back in their sockets, rub it firmly over each scarified cornea and well down under the nictitating membrane. If fluid from a capillary tube or needle is being inoculated, place the material on the cornea and massage it in with the eyelid.

The rabbit should be observed daily for the development of a keratoconjunctivitis If the test is negative, the rabbit may show slight conjunctival injection for 24 to 48 hours after inoculation but often shows no evidence of trauma If positive, a characteristic keratoconjunctivitis can occur from 12 hours to 7 days after inoculation The first signs are an injection of the conjunctiva, a slight steaminess of the cornea, which is due to the formation of numerous vesicles along the scratch marks as can be seen with a suitable magnification, and some watery exudate Within another 24 hours, the exudate becomes purulent, and photophobia becomes evident The nictitating membrane is injected and swollen Typical histologic lesions in the cornea can be seen within 24 hours after the onset of signs of infection (see below) The conjunctival exudate has been described as largely polymorphonuclear in character,[27] but a high percentage of mononuclear cells is seen in early exudates Such exudates are bacteriologically sterile and can be used for passage to the cornea of another rabbit or for intracerebral inoculation into rabbits or mice Perhaps a more reliable method for intracerebral passage of the virus is to snip off, under anesthesia, the nictitating membrane at the height of the infection, wash it in sterile saline, grind it with alundum and diluent and, after centrifuging at 2,000 r p.m , inoculate the supernatant intracerebrally into the passage animal [28]

b Intracerebral route. Aseptic tissues, such as blood, brain tissue, or cerebrospinal fluid, can be injected intracerebrally in 0 25 ml. amounts. Preliminary temperatures should be taken to avoid unsuitable animals that are already running a fever (temperatures of over 104° F. are considered significant) If virus is present the rabbit will develop a fever, usually within 24 to 72 hours after inoculation Several possible courses may then be followed: (1) The fever may last a few days without other symptoms, and the rabbit may recover. Such a rabbit will usually develop antibodies against the virus and resist a challenge dose of a known herpes virus after 3 to 4 weeks (2) The fever may persist over 105° F. for several days without any other observable symptoms, and the rabbit may die suddenly (3) The rabbit may develop signs of encephalitis, such as tremors, weakness of limbs, or the pulling of the head and neck to one side and moving in circles to that side For passage the animal should be sacrificed, the brain removed aseptically, ground in suitable

ened and edematous Otherwise, the findings resemble those found in the skin In the corium there is a pronounced inflammatory reaction with dilated capillaries and polymorphonuclear infiltration, but necrosis does not occur in an uncomplicated herpetic reaction, and healing takes place without scarring

In the rabbit cornea there is an early proliferation of the epithelial cells near the scratch marks In the center of the proliferating area, cell necrosis occurs with eventual sloughing The inclusions are best seen in the cells at the margin of the scratches or of the necrotic area In the *substantia propria*, there is considerable congestion and acute inflammatory reaction accompanied by polymorphonuclear infiltration [50] It should be noted that the granules of the polymorphonuclear cells of the rabbit tend to be quite eosinophilic and show up red with the hematoxylin and eosin stain and may therefore be mistaken at first glance for possible inclusion bodies In the rabbit brain, Da Fano and Perdrau [37] describe a chronic inflammatory lesion occurring in partially immunized rabbits

In the human brain, the following picture is described by Wolf [34a] Grossly, there is intense congestion There are foci of dusky discoloration which are swollen, soft, and friable The cortex is most involved, followed by the central white matter chiefly in relation to the cortical changes The base of the brain and the cord are relatively uninvolved Histologically, there is widespread mononuclear leptomeningitis most severe over the cortical lesions The parenchymal lesions are those of intense degeneration with relatively mild inflammation and appear to be sweeping in from the meninges The appearance is like that of an encephalomalacia due to a circulatory disturbance in which nerve cells undergo ischemic necrosis and fat-laden phagocytes flood the field The endothelial cells of the capillaries are swollen. Neuronophagia occurs Characteristic intranuclear inclusions are seen in the oligodendria and less often in the nerve cells

In the chorioallantoic membrane the pathologic changes have been studied by Crouse *et al* [20] By 20 hours after infection cell proliferation has resulted in microscopic pocks In the great majority of these cells there is a large, homogeneous, bluish, intranuclear inclusion body as stained with hematoxylin and eosin. These bodies are also Feulgen positive There are multinucleate giant cells present By 30 hours sloughing of original pocks has occurred and secondary pock formation begins At this time some of the inclusions have become shrunken and Feulgen negative. Inclusions can now be found in the mesodermal cells, together with a mild inflammatory reaction These changes increase up to 72 hours when evidence of healing appears No inclusion bodies are seen in the entodermal cells but by 48 hours the entoderm has proliferated generally and by 72 hours this has resulted in thickened folds The nucleoli of cells, before the development of the inclusion body, enlarge greatly in size, but once the inclusion body is formed, the nucleoli cannot be detected

E. Staining

The staining properties of the inclusion bodies will vary with the age of the body and perhaps the tissue in which they are studied. The classical Type A eosinophilic bodies will be more easily seen in a deep tissue than in a superficial one in which the infected cells tend to be sloughed off. Hematoxylin and eosin is probably the most widely used

D. Pathologic Specimens

These may be obtained by biopsy of characteristic superficial lesions, such as herpetic vesicles, from human autopsy material, especially the brains, from patients who died of encephalitis, and the liver, lungs, and adrenals from patients who died of generalized herpes; or from animal material such as the eye and brain or other organs of infected animals and from the pocks on the chorioallantoic membrane. The diagnostic finding in herpes virus infected tissue is the presence of the intranuclear inclusions already described (pp 317–18). These are also known as Lipschutz[32] bodies. They are characteristically demonstrable in the infected cornea of the rabbit In order to see them it is important to take the rabbit's eye out from 12 to 24 hours after the appearance of signs of conjunctivitis and before conjunctival exudate has become frankly purulent. After this time, the epithelial cells, in which the inclusions occur, have mostly sloughed off It is important to note that the time for section must be judged by the appearance of the cornea and the time after the onset of symptoms and not from the time of inoculation, since the incubation period will vary with viruses of different pathogenicity Typical inclusion bodies can be seen in the organs of infected animals or human beings. These will be found in cells of the margins of areas of necrosis or vesiculation They can also be found in the membrane and embryos of the hen's egg 24 hours after infection Anderson[33] used their presence to determine whether infection of the embryo had taken place.

Apart from the inclusion bodies, the histologic reaction varies with the tissue being examined but is, in general, of an acute inflammatory nature

In the skin and mucous membrane, the characteristic lesion is the vesicle[34] In *the skin the characteristics are clear cut while in the mucous membranes the* form of the vesicle is modified The lesion is histologically indistinguishable from those of herpes zoster and varicella Proliferation of the cells of the stratum mucosum occurs, and inter and intracellular edema develops These changes result in the development of a vesicle in the prickle cell layer which may extend down to, but does not involve, the corium The floor of the vesicle may then consist of naked papillae of the corium, and the roof of degenerating prickle cells and imperfectly *keratinized* [illegible] thickened and the cells are [illegible] -nis- [illegible] uid containing desquamated swollen epidermal cells, leukocytes, and virus giant cells containing intranuclear inclusions The giant cells can be detected by examining a Giemsa-stained smear of a scraping of the base of a herpetic vesicle *and form* a valuable aid to diagnosis[35] In the mucous membrane, the vesicle loses its fluid early through maceration and, hence, is mainly filled with fibrin The roof cells are thick-

with specific antiserum can then be compared with the end point of the suspected virus in the presence of normal serum. Since the technics of titration and neutralization overlap, that of titration will be described here whereas that of neutralization, either for suspected virus by a specific antiserum or a known herpes virus by a suspected immune serum will be described under the section dealing with serologic diagnosis of the disease.

H TITRATION OF VIRUS

Titration of the virus can be done in three ways in mice, either adult or suckling, in eggs and in tissue culture.

1. *In mice.* a In adult mice the brain of an animal moribund with herpes infection or portions of brains of several infected mice are removed aseptically, weighed, and then ground up with a suitable diluent* into a 20 per cent emulsion, this is centrifuged at approximately 2,000 r p m. for 10 minutes and the supernatant diluted serially in 10-fold dilutions† 10^{-0} to 10^{-6} With a ¼ ml tuberculin syringe and a 27-gauge ¼-inch needle, take up 0 25 ml. of the 10^{-6} dilution Under light ether anesthesia, inoculate 4 to 6 mice intracerebrally, each with 0.03 ml With the same syringe and needle, repeat the procedure with the lower dilutions in turn.

The mice should be examined daily Deaths of mice occurring within 24 hours of the inoculation should be omitted from the calculation as being due to accident The number of mice dying after this time or showing symptoms of encephalitis should be recorded each day. The test should be kept for 21 days, although if the strain is very potent, 2 weeks may be sufficient At the end of this time, the number of mice that have died or have been typically sick with encephalitis, and the number surviving and healthy should be tabulated for each dilution and a cumulative total derived, so that a 50 per cent end point can be calculated according to the formula of Reed and Muench [40]

b Titrations in 1-day-old suckling mice,[26,41] may give a sharper end point since mice die if infected There are four differences from the technic used in adult mice. (1) The upper limit of dilutions is increased to 10^{-7} because of increased susceptibility of this host, (2) 0 05 ml. of each dilution of virus is inoculated intraperitoneally, (3) deaths of mice

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* See footnote on page 319

† IN ALL VIRUS TITRATIONS, A SEPARATE PIPETTE SHOULD BE USED FOR EACH DILUTION.

and useful stain. Some authors[38] report phloxin and methylene blue as being more useful in demonstrating typical Type A inclusions in the central nervous system.

F Other Viruses to Be Ruled Out

Clinically there are only two other virus diseases likely to be confused with herpetic infection.

1. *Vaccinia* This virus causes a vesicular eruption on the atopic eczematous skin (eczema vaccinatum) which may be indistinguishable from eczema herpeticum. It causes pocks on the chorioallantoic membrane of eggs which are larger and more necrotic than those of herpes and can be distinguished for certain by finding typical cytoplasmic inclusion (Guarnieri) bodies characteristic of vaccinia or by a specific neutralization test It also causes a keratoconjunctivitis in rabbits which is indistinguishable clinically but may be distinguished histologically from that caused by herpes

2 *Coxsackie virus (Group A)* This virus causes an epidemic ulcerative pharyngitis (herpangina)[39] which may superficially resemble herpetic stomatitis. The epidemic character of the infection and the localization of the lesions to the soft palate and pharynx should suggest the correct diagnosis This can be confirmed by the failure to produce pocks on the chorioallantoic membrane with material from the lesions and the finding of lesions in suckling mice characteristic of Group A Coxsackie viruses The development of specific antibodies against Coxsackie virus can be demonstrated.

G Identification of the Virus

The virus can be identified in two ways

1. *Immunity test.* By challenging herpes-immune animals and normal controls with the virus under consideration. If the virus is herpes, the normal animals will come down with typical disease and probably die, but the immune animals will show little, if any, reaction.

2. *Neutralization test* By specific neutralization of the virus by a known herpes-immune serum

In order to demonstrate neutralization of the virus by a known specific serum, the strength of the virus in terms of infectious units must be determined by titration The end point of a suspected virus mixed

In addition to titration of the virus on the chorioallantoic membrane, some observers[13] prefer the LD_{50} of embryos after yolk sac inoculation This test is as follows:

Ten-fold serial dilutions of egg-adapted virus emulsion are made as above 0 5 ml of each dilution is inoculated into the yolk sac of each of six to eight 7-day-old embryos Eggs are candled once or twice daily Embryos dying within 48 hours are discarded as being due to nonspecific causes, but those dying between the 3d and the 12th day are recorded, and LD_{50} calculated [40] The use of time of death of embryo as an end point is not of value here since this varies with the degree of egg adaptation of the virus A comparison of sensitivity of the yolk sac death point and the chorioallantoic membrane-pock count reveals an average ratio $\frac{\text{CA pock units}}{\text{YS lethal units}}$ of 1 4, indicating slightly greater sensitivity of the pock count method.[43]

3 *In tissue culture* A series of screw cap tubes containing sheets of HeLa cells in maintenance medium are prepared Ten-fold serial dilutions of tissue culture adapted virus are made and 0 1 ml of each dilution inoculated into each of 2 tubes The tubes are incubated in stationary racks at 37° C for 7 days, and the number of tubes showing cytopathogenicity are recorded each day The titer is calculated as the reciprocal of the highest dilution in which cytopathogenicity is observed

I Preservation and Destruction

1 *Preservation*

a In animal tissue Virus in animal tissue is relatively stable It can be preserved in 50 per cent glycerol at 4° to 8° C for 6 months and up to 18 months when desiccated from the frozen state [44] In 50 per cent glycerol at −20° C., infected mouse brain has been kept for 18 months in this laboratory with a drop in 50 per cent end point titer from 10^{-4} to 10^{-2} Suspensions can be well preserved at −70° C after shell freezing in sealed glass ampules *

b Egg material Egg adapted material can be [illegible]

* When storing the virus in a dry-ice cabinet it is important to use glass sealed ampules The use of rubber stoppers allows entrance of CO_2 into vials which, in some cases, causes deterioration of virus

occurring within 36 hours are omitted from calculation, and thereafter deaths and survivors are tabulated as above for calculation of 50 per cent end point; (4) the test is observed for one week.

2. *In eggs.* Since the virus produces characteristic pocks on the chorioallantoic membrane, this provides a convenient and inexpensive method of titrating the virus. One or more chorioallantoic membranes infected with the herpes simplex are weighed and ground in suitable diluent* with sterile alundum to a concentration of 10 to 20 per cent. This is centrifuged at approximately 2,000 r.p.m. for 10 minutes and the supernatant diluted in decimal steps to 10^{-6} or 10^{-7} in the above diluent. With the same ¼ or ½ ml. tuberculin syringe and a No. 27 gauge needle, 0.05 ml. of each dilution, starting with the highest, is inoculated onto the chorioallantoic membrane of each of four to six 12- to 13-day-old eggs, all of which are harvested after 40 to 48 hours as described above. In the lower dilutions, the individual pocks may not be distinguishable, the whole membrane being opaque, boggy, and edematous, with higher dilutions, however, discrete pocks will appear in numbers that can be counted, until an end point is reached beyond which no pocks are visible and the membranes have their normal thin, glistening appearance.

There are certain difficulties in making the counts. Distribution of the virus over the membrane is often not good so that the pocks are confluent and therefore uncountable. In lower dilutions such confluent membranes indicate a heavy infection. One such membrane may occur, however, at a high dilution in which the other three membranes are negative or contain only a few pocks. If membranes that are greatly out of line are discarded and the rest averaged, however, the results are sufficiently accurate for diagnostic purposes. The reproducibility of the method in our hands is with ± 1 log,[42] and Beveridge and Burnet[25] calculated that, using 4 to 6 eggs per dilution, they could get end points within ± 50 per cent of the true mean.

In working out the final titer of the virus from membrane counts, two methods are current. One calculates the infectious units/ml. of virus as follows. With as many dilutions as it is possible to obtain good counts, the pocks are counted and averaged. This number is multiplied by the last dilution in which pocks are seen. The product thus derived is again multiplied by the reciprocal of the fraction of a ml. used for inoculation; for example, if there were an average of 5 pocks at 10^{-6} and 60 pocks at 10^{-5} (lower dilutions being confluent), then the final count would be $5.5 \times 10^{6} \times 20$ (0.05 ml. being inoculated) $= 1.1 \times 10^{8}$ infectious units/ml. The second calculates the ID_{50} (Reed and Muench) using presence or absence of pocks on the membrane to indicate infection or lack of infection.

* See footnote on page 319

f Gentian violet This is relatively harmless to the virus. An infected rabbit brain emulsion, exposed to 1 per cent gentian violet for 18 hours at 4° to 8° C and then washed free of the dye, still contains sufficient virus to infect a rabbit on intracerebral injection [44] If an infected chorioallantoic membrane emulsion is exposed to 1:5,000 gentian violet for 30 minutes, however, and if the whole mixture is inoculated onto chorioallantoic membranes of other eggs, the virus will have lost 95 to 99 per cent of its potency No reduction of potency is found when the gentian violet concentration is reduced to a concentration of 1.50,000.[9]

g Temperature The effect of this varies with the state of the virus. In the moist state it is destroyed at 52° C after 30 minutes and at 37° C. after 24 hours although still potent after 6 to 8 hours, in the desiccated state, it resists 90° C for 30 minutes but is destroyed by heating at 100° C. for 30 minutes [44]

h Miscellaneous

(1) Amos[49] reported the inactivation of herpes virus by acid and alkali phosphatase but no inactivation with trypsin, pepsin, ribonuclease, ptyalin, or lipase.

(2) In the presence of egg white the virus is inactivated rapidly at 37° C [23a]

The observations in this section were made on virus in the form of tissue emulsion unless otherwise indicated

III. SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE

A Collection of Material

Blood should be collected with aseptic precautions, preferably within 4 days after the onset of a clinical syndrome suspected of being herpetic in origin, allowed to clot, and the serum separated. This should be repeated after an interval of 2 to 3 weeks The initial and subsequent sera are used for serologic tests to demonstrate the appearance of antibody against herpes or to prove the absence of such a phenomenon. The sera can be used fresh or after storage at 20° C. They can be shipped in sterile, rubber-stoppered tubes in regulation mailing cartons. No preservatives should be added

B Neutralization Test

1. *In mice* Satisfactory tests are as follows:

a. Adult mice. A 20 per cent suspension of infected mouse brain is

or at −70° C for at least 10 months Also in this laboratory it has been possible to maintain unground whole infected CAM in 15 per cent glycerol at −20° C. for 21 months. Fifty per cent glycerol was less effective in preserving the virus

c Purified virus particles.[10] Purified suspensions of virus are stable in distilled water and 0.25 M sucrose but unstable in allantoic fluid. A pH of over 7 0 increased the stability of the virus in ionic solutions which, however, are less stabilizing than nonionic.

d Solutions The virus in any form deteriorates in physiologic saline, but this can be overcome by buffering the saline to a pH of *7 2* and adding 10 per cent rabbit[47] or horse serum,[10] yolk 0.1 per cent[23a] or 0 5 per cent gelatin.[23] It also keeps better in nutrient broth A low oxygen tension, such as attained by adding cysteine to the preserving fluid, also helps to preserve the virus.[47]

2. *Destruction.*

a. Bile. The virus is destroyed by bile.[48]

b Organic solvents [44] The virus is destroyed in the wet state by exposure to alcohol, chloroform, or ether for 18 hours at 4° C. In the dry state, however, it resists the action of absolute alcohol and ether under similar conditions of exposure.

c. Phenol. The virus resists 1 per cent concentration of phenol for 15 minutes at room temperature although it is destroyed by the same concentration after exposure for 3 days.[24] Exposure to 5 per cent concentration for 18 hours at 4° C. also destroys the virus [44]

d. Potassium *permanganate* [48] 1 *1,000 concentration* destroys the virus in 1 hour at room temperature

e. Quaternary ammonium cationic detergents.[9] Several of these destroy the virus in vitro. Of those commonly available, Ceepryn (cetyl pyridinium chloride) and Zephiran (benzalkonium chloride) are both effective. Of the egg-adapted virus, approximately 10^6 infectious units are destroyed by either agent in a concentration of 1:10,000 in 30 minutes at room temperature. However, the virus is not destroyed under similar conditions by a concentration of 1:25,000 Zephiran or 1:50,000 [illegible] *There is no*

[illegible] minutes

[illegible] infected

[illegible] ncentra-

tion of 1·5,000 leads to a decrease in titer from 10^{-5} to 10^{-1} as tested by intracerebral inoculation of adult mice.

activity is important for study of patients, the following routine is used in this laboratory.

A concentration of virus is chosen which will give an easily determined number of plaques (± 100) on the membrane This is incubated with an equal quantity of the serum to be tested and a control serum, the sera being inactivated at 60° C for 20 minutes The test human sera are used undiluted and diluted in 4-fold steps to 1 256. The control normal rabbit serum is used diluted 1 16 or more, since undiluted rabbit serum is found at times to decrease the pock count The time of incubation is not emphasized since it has been shown that neutralization can take place within 1 minute [52] However, recently, it has been stated that incubation for 1 hour at 37° C is required for best results.[43] 0 05 ml. of each mixture is then inoculated onto the chorioallantoic membrane of each of four to six 12- to 13-day-old eggs using a different syringe for each mixture After 40 hours' incubation at 36° C the membranes are removed, and the pocks counted as described above. The antibody titer of each serum is recorded as the highest dilution of that serum which reduces the pock count below 50 per cent of that found with control serum

Reliable neutralization tests using yolk sac inoculation and the death of the embryo as the end point have been described.[43] Two types may be used, either the constant serum-virus dilution method or the constant virus-serum dilution method (*a*) In the former, test sera and control, inactivated by heating at 56° C. for 30 minutes, are diluted routinely 1.30, or more as indicated, and thoroughly mixed with an equal quantity of egg-adapted virus in dilutions of 10^{-1} to 10^{-5}. After incubation at 37° C for 60 minutes, 0 5 ml of each serum-virus mixture is inoculated into the yolk sac of each of six to eight 7-day-old embryonated eggs. These are incubated at 36° C and candled once to twice daily Embryos dying before 48 hours are discarded Those dying between the 3d and 12th day are recorded and the LD_{50} calculated. (*b*) In the latter a constant concentration of virus (3 to 30 LD_{50}) is mixed with falling 2- to 4-fold dilutions of serum to be tested All sera including normal rabbit control are inactivated at 56° C for 30 minutes The mixtures are allowed to incubate for 1 hour at 37° C, and then 0 5 ml of each mixture is inoculated into the yolk sac of each of six to eight 7-day-old embryos as above. The end points can be recorded as the reciprocal of the serum dilution affording 50 per cent protection = PD_{50} Either of these two methods appears to be satisfactory

prepared and a 1.5 dilution of supernate is made in suitable diluent.* Decimal dilutions are then made up to 10^{-5} giving concentrations of 1:5 up to 1.500,000 in 0 15 ml. These, when added to equal amounts (0 15 ml) of undiluted test or control sera, inactivated by heating at 60° C. for 20 minutes, give final concentrations of 1:10, 1:100, etc, up to 1 1,000,000. The mixtures of virus dilution and serum are incubated for 1 hour at room temperature or 4° C At the end of this time each mixture is injected intracerebrally (0.03 ml /mouse)† into groups of 4 to 6 mice, which are observed daily over a period of 21 days for death and/or central nervous system signs.

b. Suckling mice A neutralization test can be carried out in newborn mice.[41] Test and control sera, inactivated by heating at 56° C. for 30 minutes, are diluted in serial 4-fold dilutions Each dilution is mixed with a constant quantity of virus (25 to 50 LD_{50}) The mixture is incubated at room temperature for 5 to 10 minutes, and the 0 05 of each dilution is inoculated intraperitoneally into each of 6 to 8 mice per dilution Deaths of mice occurring within 36 hours are not included in test, being considered nonspecific in character. Most specific deaths occur between 2 and 5 days, and the end point is a sharp one between death and survival Animals should be observed for 1 week.

Dead and living mice are cumulated for each dilution, and LD_{50} titers can be calculated *for each serum* [40] Neutralization indexes can then be determined by the standard methods [50]

2 *In eggs* An "all or none" neutralization test was described by Burnet and Lush as follows.[51] Undiluted test sera with control, inactivated at 56° C for 20 minutes, are mixed with equal volumes of virus diluted sufficiently to give a pock count of approximately 200 pocks After incubation for 1½ to 2 hours at 4° C , 0 05 ml. of each mixture is inoculated onto the chorioallantoic membrane of four 12-day-old eggs After a 40-hour incubation, a pock count is made, and the results recorded as percentage of pocks found with the diluted virus-test serum mixture as compared with those found with the diluted virus-control serum mixture Positive sera, as a rule, cause a reduction of pocks to below 0 1 *per cent of control whereas with negative sera*, the pock count is not less than 30 per cent of the control

Since a quantitative comparison of the amount of neutralization

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* See footnote on page 319

† Use different syringe for each dilution

activity is important for study of patients, the following routine is used in this laboratory.

A concentration of virus is chosen which will give an easily determined number of plaques (± 100) on the membrane. This is incubated with an equal quantity of the serum to be tested and a control serum, the sera being inactivated at 60° C for 20 minutes The test human sera are used undiluted and diluted in 4-fold steps to 1 256 The control normal rabbit serum is used diluted 1 16 or more, since undiluted rabbit serum is found at times to decrease the pock count The time of incubation is not emphasized since it has been shown that neutralization can take place within 1 minute[52] However, recently, it has been stated that incubation for 1 hour at 37° C. is required for best results[43] 0 05 ml of each mixture is then inoculated onto the chorioallantoic membrane of each of four to six 12- to 13-day-old eggs using a different syringe for each mixture After 40 hours' incubation at 36° C the membranes are removed, and the pocks counted as described above The antibody titer of each serum is recorded as the highest dilution of that serum which reduces the pock count below 50 per cent of that found with control serum

Reliable neutralization tests using yolk sac inoculation and the death of the embryo as the end point have been described.[43] Two types may be used, either the constant serum-virus dilution method or the constant virus-serum dilution method. (*a*) In the former, test sera and control, inactivated by heating at 56° C for 30 minutes, are diluted routinely 1 30, or more as indicated, and thoroughly mixed with an equal quantity of egg-adapted virus in dilutions of 10^{-1} to 10^{-6}. After incubation at 37° C for 60 minutes, 0 5 ml of each serum-virus mixture is inoculated into the yolk sac of each of six to eight 7-day-old embryonated eggs These are incubated at 36° C and candled once to twice daily Embryos dying before 48 hours are discarded Those dying between the 3d and 12th day are recorded and the LD_{50} calculated (*b*) In the latter a constant concentration of virus (3 to 30 LD_{50}) is mixed with falling 2- to 4-fold dilutions of serum to be tested All sera including normal rabbit control are inactivated at 56° C. for 30 minutes The mixtures are allowed to incubate for 1 hour at 37° C, and then 0 5 ml of each mixture is inoculated into the yolk sac of each of six to eight 7-day-old embryos as above. The end points can be recorded as the reciprocal of the serum dilution affording 50 per cent protection = PD_{50} Either of these two methods appears to be satisfactory.

3. *In tissue culture.* Incubate ± 100 ID_{50} doses of virus in maintenance medium with equal quantities of appropriate dilutions of test and control sera inactivated at 56° C. for 30 minutes. Inoculate 0.1 ml of each mixture into each of 2 tissue culture tubes containing sheets of HeLa cells in 1.0 ml of maintenance medium. Incubate in stationary racks for 7 days. Tubes should be read daily The titer is calculated as the reciprocal of the dilution of serum that prevents the occurrence of lesions in the cell sheets.

C. Complement Fixation Test

Since the first edition of this book, several studies on the use of the complement fixation test as a diagnostic tool have appeared employing antigens derived from infected eggs[41,53,54,55] An important factor in obtaining a good CF antigen seems to be the use of a recently adapted strain. The difficulties encountered that prevented the widespread use of this test may have stemmed from the use of standard laboratory strains that had been passaged frequently in eggs.

The following technic as described by Sosa-Martinez and Lennette[41] has been found to be very satisfactory in our laboratory.

Seventy to ninety 8-day-old embryos (enough to supply 50 to 60 gms. of chorioallantoic membranes) are inoculated via the yolk sac with 50 LD_{50} doses of infected yolk sac suspension. Embryos are incubated at 37° C. Eggs dying before 40th hour are discarded. Between 40th to 50th hours, eggs are candled twice, and those recently dead or sluggish are placed in refrigerator (+ 4° C.). At 50th hour, the remaining eggs are placed in refrigerator and chilled from 4 to 18 hours before harvesting. Chorioallantoic membranes and amniotic sacs are removed, thoroughly rinsed with saline, and drained in petri dishes The membranes are weighed, ground with alundum, and buffered saline, pH 7.2, is added to make a 25 per cent suspension (W/V). After centrifugation for 20 minutes at 2,000 r.p m., the supernatant is placed in tubes in 8 to 10 ml. amounts and stored in the dry-ice chest for future use.

For the test the necessary amount is thawed, centrifuged at 2,000 r.p.m. for 20 minutes, and the supernate removed and titrated by box titration method. The unit has a titer range from 1:4 to 1:8 of the 25 per cent suspension. The titration employs 0 2 ml. of falling dilutions of serum (starting with 1:8 dilution) 2 units of antigen in 0.2 ml, 2 full units of complement in 0 2 ml., incubation for 18 hours (overnight) at 4° C. After a final addition of 0 5 ml. of sensitized cells (equal volume of 2 per cent sheep erythrocytes and 2 units of hemolysin), the test is

incubated at 37° C for 10 to 15 minutes until the controls clear. The tubes are then removed from the bath, held at room temperature for 20 minutes, and then read The end point is taken at 3 plus complement fixation.

Normal chorioallantoic membrane and amniotic sacs prepared in the same way are used as controls in the same dilutions.

Contaminated or old serums (kept in the refrigerator for 2 years) may give nonspecific results with antigens at the concentration used in the test For the test, sera are inactivated at 62° C. for 15 minutes.

D Skin Test

Several investigators have demonstrated that a positive skin test can be demonstrated in individuals having neutralizing antibodies in their blood stream Either chorioallantoic membrane suspension [56], amniotic,[57] chorioallantoic fluid,[58] or a combination of amniotic and allantoic fluids[59] has been used as source of skin test antigen. The contained virus has been destroyed by heating at 65° C. to 70° C for 2 hours on 2 successive days,[56,57] or at 56° C [58] for 2 hours, or by ultraviolet light irradiation for approximately 2½ times as long as required to destroy all detectable virus [59] The technic involves the inoculation of 0 1 ml of the test antigen intradermally on the volar surface of the one forearm and 0 1 ml of control material from uninfected eggs (chorioallantoic membrane, amniotic and/or allantoic fluid) in a similar manner on the volar surface of the other.

A positive skin test is described as erythema and induration of greater than 5 mm in average diameter, the control being completely negative or less than 5 mm in maximum diameter Nonspecific reactions appear to be less common with fluid than with membrane antigens In older persons the skin test reaction may be negative despite the presence of circulating antibodies [9] The antigen is stable at −20° C for many months Evidence suggests that the active agent is a soluble antigen separable from the infectious virus [59] It is necessary, however, to prepare the antigen from material with a high infectivity titer, greater than 10^5 infectious units/ml in order to obtain good results

IV REFERENCES

1. Löwenstein, A Aetiologische Untersuchungen über den fieberhaften Herpes *München med Wchnschr*, 66 769–770, 1919

2. Gruter, W Experimentelle und klinische Untersuchungen über den sogenannten Herpes corneae *Ber ü d Versamml d Ophth Gesellsch* 2d ed 42 162–167, 1920

3 Scott, T F McN Diseases Caused by the Virus of Herpes Simplex Chapter 25 in Rivers, T M, *Viral and Rickettsial Infections of Man* 2d ed, Philadelphia J B Lippincott Company, 1952, pp 491–502
4 Zuelzer, W W, and Stulberg, C S Herpes Simplex Virus as the Cause of Fulminating Visceral Disease and Hepatitis in Infancy *Am. J. Dis Child*, 83 421–439, 1952.
5. Buddingh, G J., Schrum, D I, Lanier, J C, and Guidry, D J Studies of the Natural History of Herpes Simplex Infections *Pediatrics*, 11·595-610, 1953.
6 Elford, W J, Perdrau, J R, and Smith, W. Filtration of Herpes Virus through Graded Collodion Membranes *J Path and Bact*, 36 49–54, 1933.
7 Ward, H K, and Tang, F F Note on Filtration of Virus of Herpetic Encephalitis and of Vaccinia *J Exper Med*, 49 1–4, 1929
8 Bedson, S P Some Obser[illegible] *Brit J.* [illegible]
9 [illegible]
10 [illegible] Virus *J* [illegible]
11 [illegible] al Charge [illegible]
12 [illegible] *Soc.* [illegible]
13 [illegible] ter [illegible]
14 [illegible] 'en- [illegible] 58–366, 1934
15 Evans, A S, and Melnick, J L Electron Microscope Studies of the Vesicle and Spinal Fluids from a Case of Herpes Zoster *Proc. Soc Exper. Biol and Med*, 71 283–286, 1949
16 Coriell, L L, Rake, G, Blank, H, and Scott, T F McN Electron Microscopy of Herpes Simplex *J Bact*, 59 61–68, 1950.
17 Morgan, C, Ellison, S A, Rose, H M, and Moore, D H Structure and Development of Viruses as Observed in the Electron Microscope I Herpes Simplex Virus *J Exper Med*, 100 195–202, 1954
18 Reagan, R L, Sansone, F M, Moore, S, and Brueckner, A L. Electron Microscopic Studies of Herpes Simplex Virus after Propagation in the Swiss Albino Mouse *Texas Rep Biol and Med*, 11 79–82, 1953
19 Scherer, W F The Viral Range In Vitro of a Malignant Human Epithelial Cell (Strain HeLa, Gey) I Multiplication of Herpes Simplex, Pseudorabies, and Vaccinia Viruses *Am J Path*, 30 1057–1073, 1954
19a [illegible]
20 [illegible]
21. [illegible] Inclusion Bodies *J Infect Dis*, 91 63–71, 1952
22. Cowdry, E V The Problem of Intranuclear Inclusion in Virus Diseases *Arch Path*, 18 527–542, 1934
23. Coriell, L L, Blank, H, and Scott, T F McN Isolation of Herpes Simplex Virus on the Chorioallantoic Membrane *J Lab and Clin Med*, 34 402–408, 1949
23a Yoshino, K Personal communication
24 Levaditi, C, and Harvier, P Étude expérimentale de l'encéphalite dite léthargique *Ann Inst Pasteur*, 34 911–972, 1920
25. Beveridge, W I B, and Burnet, F. M. The Cultivation of Viruses and Rickettsiae in the Chick Embryo *Med[illegible] Report Series No. 256*, [illegible] c) pp 30–33.
25a [illegible] ers, T. M, *Viral and* [illegible] Lippincott Company, [illegible]

26 Kilbourne, E D, and Horsfall, F L, Jr Studies of Herpes Simplex Virus in Newborn Mice *J Immunol*, 67 321–329, 1951
27 Friedenwald, J S Studies in the Virus of Herpes Simplex *Arch Ophth*, 52 105–131, 1923
28 Steigman, A J, and Scott, T F McN Herpes Simplex Virus Simple Method for Adapting Human Strains to Mice *Lancet*, 1 761–762, 1942
29 Zarafonetis, C J D, Smadel, J E, Adams, J W, and Haymaker, W. Fatal Herpes Simplex Encephalitis in Man *Am J Path*, 20 429–445, 1944
30 Bedson, S P, and Crawford, G J Immunity in Experimental Herpes *Brit J Exper Path*, 8 138–147, 1927
31 Fl [illegible]
32 Li [illegible]
428–482, 1921
33 Anderson, K Pathogenesis of Herpes Simplex Virus Infection in Chick Embryos *Am J Path*, 16 137–156, 1940
34 Scott, T F McN, Blank, H, Coriell, L L, and Crouse, H Pathology and Pathogenesis of the Cutaneous Lesions of Variola, Vaccinia, Herpes Simplex, Herpes [illegible]
34a [illegible]
35 [illegible]
36 Van Rooyen, C E, and Rhodes, A J *Virus Diseases of Man* 2d ed New York Thomas Nelson and Sons, 1948, p 176
37 DaFano, C, and Perdrau, J R Chronic or Subacute Herpetic Meningoencephalitis in the Rabbit with Some Observations on Calcification *J Path and Bact*, 30 67–95, 1927
38 Whitman, L, Wall, M J and Warren, J Herpes Simplex Encephalitis A Report of Two Fatal Cases *J A M A*, 131 1408–1411, 1946
39 Huebner, R J, Cole, R M, Beeman, E A, Bell, J A, and Peers, J H Herpangina Etiological Studies of a Specific Infectious Disease *J A M A*, 145 628–633, 1951
40 [illegible] Estimating 50 Per Cent End Points
41 [illegible] s on a Complement Fixation Test for
42 Scott, T F McN, Coriell, L L Blank, H, and Gray, A The Growth Curve of the [illegible]
43 [illegible]
44 Holden, M Nature and Properties of Virus of Herpes *J Infect Dis*, 50 218–236, 1932
45 Speck, R S., Jawetz, E, and Coleman V R Studies on Herpes Simplex Virus I The Stability and Preservation of Egg-Adapted Herpes Simplex Virus. *J. Bact*, 61 253–258, 1951
46 [illegible]
47 [illegible] 17 343–355 1929.
48 Levaditi, C, Harvier, P, and Nicolau, S Étude expérimentale de l'encéphalite dite "léthargique" *Ann Inst Pasteur*, 36 63–101, 1922

3 Scott, T F McN Diseases Caused by the Virus of Herpes Simplex Chapter 25 in Rivers, T M, *Viral and Rickettsial Infections of Man* 2d ed Philadelphia [illegible]

4 [illegible] lex Virus as the Cause of Fulminat- [illegible] *Am. J. Dis Child*, 83 421–439, [illegible]

5 Buddingh, G J, Schrum, D. I, Lanier, J C, and Guidry, D J. Studies of the Natural History of Herpes Simplex Infections *Pediatrics*, 11 595–610, 1953

6 Elford, W J, Perdrau, J R, and Smith, W Filtration of Herpes Virus through Graded Collodion Membranes *J Path and Bact*, 36 49–54, 1933.

7 Ward, H K, and Tang, F F Note on Filtration of Virus of Herpetic Encephalitis and of Vaccinia *J Exper Med*, 49 1–4, 1929

8 Bedson, S P Some Observations Bearing on Size of Herpes Virus Particles *Brit J Exper Path*, 8 470–479, 1927.

9 Unpublished observations

10 Munk, K., and Ackermann, W W Some Properties of Herpes Simplex Virus *J Immunol*, 71 426–430, 1953

11 [illegible] g Electrical Charge [illegible] '9.

12 [illegible] *Compt rend soc* [illegible]

13 Taniguchi, T, Hosokawa, M, Kuga, S, and Masuda, Z Virus of Herpes and Zoster. *Jap J Exper Med*, 12 101–104, 1934

14 Hertzberg, K Viktoriablau zur Farbung von filtrierbarem Virus (Pocken-Varizellen-Ektromelia-und Kanarienvogelvirus) *Zentralbl f Bakt Abt 1, Orig*, 131 358–366, 1934

15 Evans, A S, and Melnick, J L Electron Microscope Studies of the Vesicle and Spinal Fluids from a Case of Herpes Zoster *Proc. Soc. Exper Biol and Med*, 71 283–286, 1949

16 Coriell, L L, Rake, G, Blank, H, and Scott, T F McN Electron Microscopy of Herpes Simplex *J Bact*, 59 61–68, 1950

17 Morgan, C, Ellison, S A, Rose, H M, and Moore, D H Structure and Development of Viruses as Observed in the Electron Microscope I. Herpes Simplex Virus *J Exper Med*, 100 195–202, 1954.

18 Reagan, R L, Sansone, F M Moore, S, and Brueckner, A L Electron Microscopic Studies of Herpes Simplex Virus after Propagation in the Swiss Albino Mouse *Texas Rep Biol and Med*, 11 79–82, 1953.

19 Scherer, W. F The Viral Range In Vitro of a Malignant Human Epithelial Cell (Strain HeLa, Gey) I Multiplication of Herpes Simplex, Pseudorabies, and Vaccinia Viruses *Am J Path*, 30 1057–1073, 1954

19a Scherer, W F The Utilization of a Pure Strain of Mammalian Cells (Earle) for the Cultivation of Viruses in Vitro *Am J Path*, 29 113–138, 1953

20 Crouse, H V, Coriell, L L, Blank, H, and Scott, T F McN Cytochemical Studies on the Intranuclear Inclusion of Herpes Simplex *J Immunol*, 65 119–128, 1950

21 Wolman, M, and Behar, A Cytochemical Evidence for the Nature of Herpes Simplex Inclusion Bodies *J Infect Dis*, 91 63–71, 1952

22 Cowdry, E V The Problem of Intranuclear Inclusion in Virus Diseases *Arch Path*, 18 527–542, 1934

23 Coriell, L L, Blank, H, and Scott, T F McN Isolation of Herpes Simplex Virus on the Chorioallantoic Membrane *J Lab and Clin Med*, 34 402–408, 1949

23a Yoshino, K Personal communication

24 Levaditi, C, and Harvier, P Étude expérimentale de l'encéphalite dite léthargique *Ann Inst Pasteur*, 34 911–972, 1920

25 Beveridge, W I B, and Burnet, F M The Cultivation of Viruses and Rickettsiae in the Chick Embryo *Medical Research Council, Special Report Series No 256*, London, His Majesty's Stat Off, 1946 *a*) p 16 *b*) p 30 *c*) pp 30–33.

25a. Buddingh, G J Chick-Embryo Technics Chapter 5 in Rivers, T M, *Viral and Rickettsial Infections of Man* 2d ed Philadelphia J B Lippincott Company, 1952, pp 109–125

VARIOLA AND VACCINIA

C. HENRY KEMPE, M.D.

Assistant Professor of Pediatrics, University of California School of Medicine, In Charge, The E Charles Fleischner Memorial Laboratory at the George Williams Hooper Foundation, San Francisco

49 Amos, H The Inactivation of Herpes Simplex by Phosphatase Enzymes *J Exper Med*, 98 365–372, 1953

50 [illegible]

51 [illegible]

52 [illegible], M [illegible], and [illegible], J F Quartitative Studies on Infectivity of Virus of Herpes Simplex for Chorioallantoic Membrane of Chick Embryo, Together with Observations on Inactivation of Virus by Its Specific Antiserum *J Immunol*, 37 383–411, 1939

53 Hayward, M E Serological Studies with Herpes Simplex Virus *Brit J Exper Path*, 30 520–529, 1949.

54 Gajdusek, D C, Robbins, M L, and Robbins, F C Diagnosis of Herpes Simplex Infections by the Complement Fixation Test *J A M A*, 149 235–240, 1952

55 Dudgeon, J A A Complement Fixation Test for Herpes Simplex Infections *J Clin Path*, 3 239–247, 1950

56 Nagler, F P O A Specific Cutaneous Reaction in Persons Infected with the Virus of Herpes Simplex *J Immunol*, 48 213–219, 1944

57 Nagler, F P O A Herpes Skin Test Reagent from Amniotic Fluid *Australian J Exper Biol and M Sc*, 24 103–105, 1946

58 Rose, H M, and Molloy, E Cutaneous Reactions with the Virus of Herpes Simplex *J Immunol*, 56 287–294, 1947

59 Jawetz, E, Coleman, V, and Allende, M F Studies on Herpes Simplex Virus II A Soluble Antigen of Herpes Virus Possessing Skin-Reactive Properties *J Immunol*, 67 197–205, 1951

abruptly and the eruptive stage begins Within the next 3 to 5 days the eruption passes rapidly through the formation of papules (deep-seated with a shotlike feel), and vesicles, to the formation of pustules (frequently umbilicated when fully developed) The temperature tends to rise as the pustules develop, varying with the severity of the disease In cases in which death occurs 10 days or more after the onset, the secondary rise in temperature of the patients may be pronounced, although in milder cases which survive it may be slight or absent There may be a moderate leukocytosis in this stage The distribution of lesions is peripheral for they are more numerous on the face, hands, and feet than on the trunk Variola lesions are commonly seen on the palms of the hands, the soles of the feet, and the mucosal surfaces of the mouth and throat and are approximately in the same stage of development at any one time In severe cases the lesions may be confluent

The final or scabbing stage is usually initiated on the 12th to 14th day of the disease As the pustules heal, crusts form and gradually fall Healing is usually complete 3 to 6 weeks after onset of the disease

There is also a fulminating, hemorrhagic type of variola frequently misdiagnosed as meningococcemia or blood dyscrasia that is a highly fatal form and of particular diagnostic significance It is characterized by the onset of petechial or massive hemorrhagic eruptions during the pre-eruptive febrile period With the failure of development of characteristic vesicles or pustules, it runs a rapidly fatal course within 4 days In addition to this early type of hemorrhagic smallpox, a later type is recognized in which hemorrhagic manifestations continue for a period of 10 to 12 days, until death occurs Continued high fever, delirium, and hematuria are common observations

Transmission of variola is usually through association with persons suffering from the disease, although such contact need not be intimate Indirect infection frequently occurs through articles contaminated by contact with patients, such as bed sheets or bedpans Patients are infective from the first symptoms until all scabs and crusts have been shed

B Pathogenesis of Variola

Little is known of the pathogenesis during the interval from infection until the onset of fever 12 days later If variola is analogous to mousepox, the classic experiments of Fenner[1] suggest that the virus enters through the mucosa of the respiratory tract, quickly passes to the respiratory lymph glands and, for a very short time, enters the blood stream early in the incubation period Virus particles presumably become quickly phagocytized by the cells of the reticulo-endothelial system throughout the body. During the rest of the incubation period, intracellular multiplication probably continues in the cells of the reticulo-endothelial system until the virus again enters the blood stream—this time more massively, and simultaneous with the onset of the febrile phase of the disease

While these steps have not yet been demonstrated in human cases of variola, the viremia coincident with the onset of the febrile

- C Serologic and Immunologic Methods of Diagnosis
  - 1 Hemagglutination-inhibition test
    - a Reagents
    - b The test
    - c. Interpretation
  - 2 Neutralization test
    - a Reagents
    - b *The test*
    - c Interpretation
  - 3 Complement fixation test
    - a. Reagents
    - b The test
    - c. Interpretation
- V References

I. INTRODUCTION

Variola (smallpox), vaccinia, and cowpox are exanthematous infections caused by immunologically closely related but distinct viruses The variola virus is transmitted from man to man and has no animal reservoir or vector The disease is world-wide in distribution and when introduced into a community its incidence largely depends on the number of susceptible inhabitants It is most prevalent in southeast Asia, Central Africa, and Central and South America and occurs in sporadic and epidemic forms—with the highest incidence during the winter and spring Cowpox is an infection of cattle and, in its naturally acquired form, is an occupational disease of milkers handling infected cows Vaccinia virus strains are laboratory strains presumed to have been derived from cowpox and skin-adapted by calf and rabbit skin passages Vaccinia virus is now universally utilized for artificial, active immunization against smallpox

In the course of routine vaccinations occasional serious complications occur, with widespread involvement of the skin such as eczema vaccinatum, generalized vaccinia, and vaccinia necrosum (progressive vaccinia). These complications have high mortality rates and are of considerable diagnostic, therapeutic, and medicolegal significance.

II. VARIOLA

A. Clinical Manifestations

The pre-eruptive stage is initiated about 12 days after exposure to variola virus (range 8 to 22 days) and is characterized by sudden onset of high fever, prostration, and generalized aching, which is particularly severe in the lumbar region A moderate leukopenia is seen early in the disease.

These symptoms continue from 3 to 4 days, after which the temperature drops

membrane lesions provides the most common source for diagnostic virus isolation.

Viremia at the onset of the febrile illness leads to infection of the capillary endothelium of the subcutaneous vessels with perivascular cellular infiltration and extensive hemorrhages The maculopapular lesions on the 3d or 4th day of disease represent the early vascular reaction to infection of the epithelium Scrapings of these lesions, involving the lower epithelial layers, show many elementary bodies in stained smears.

In the vesicular stage widespread inflammatory reaction to necrosis of previously infected epithelial cells is evident, and demonstration of virus particles within cell cytoplasm can be readily accomplished with smears taken from the base of smallpox vesicles

III COWPOX AND VACCINIA

A Cowpox

Cowpox is a generally mild occupational disease of milkers handling infected cows, which results in the development of local lesions of the hands Regional lymphadenitis, malaise, and moderate elevation in temperature are usually present Lesions are similar to those seen in typical primary vaccination and require 3 to 5 weeks to heal Generalized eruptions occasionally occur in unvaccinated individuals

B. Vaccinia

Primary vaccinia is usually the result of vaccination against smallpox with infected calf lymph It may occur accidentally in laboratory workers, especially among those engaged in the production of calf lymph vaccine, when the eyes, lips, or fingers may become affected After inoculation, a small papule appears on approximately the 3d day, becomes vesicular on the 6th or 7th day, and pustular in

As an occasional complication of primary vaccination, wide "seeding" of the vaccinia virus may occur (generalized vaccinia) More commonly, children with eczema become accidentally infected (eczema vaccinatum), usually from a vaccinated sibling Rarely, vaccinia progressively involves all skin adjacent to the vaccination site, leading to ultimate death (vaccinia necrosum)

Generalized vaccinia occurs in children and adults who have no predisposing skin lesions as a complication, usually at the height of the primary vaccinia "take" or shortly thereafter (past the 13th day after vaccination) These complications are probably instances of massive viremia with skin localization, which may be based on some fault in the immune mechanism[5] The course of such cases of generalized vaccinia is not usually fatal

In eczema vaccinatum innumerable lesions are found over the areas of the skin

illness has been consistently substantiated. The work of Downie *et al*[2] indicates that the degree and duration of this viremia varies with the severity of the illness. In patients who do not die of the disease, virus can be isolated from blood only on the 1st and 2d day of disease In patients whose disease is ultimately fatal, virus has been isolated with more frequency and at a later time in the disease (3d to 8th day). Whenever antigen is detected in the blood by the complement fixation technic, a fatal outcome may be predicted.[3] The demonstrable presence of antigen in the blood, ease of primary isolation of virus in the first 2 days, and isolation of the virus after the 2d day, all reflect massive viremia and a grave prognosis.

Neutralizing and hemagglutination-inhibiting antibodies rise before complement-fixing antibodies in variola infections Neutralizing antibodies can be demonstrated in previously vaccinated patients after the 3d day of disease, and after the 5th or 6th day in unvaccinated individuals.

Hemagglutination-inhibiting antibodies can be demonstrated on the 3d day of disease, almost coincident with the appearance of the eruption. During the subsequent week there is a rapid rise in antibodies. In unvaccinated smallpox patients, the occurrence of hemagglutination-inhibiting antibodies of moderately high titer (1 160 or above) in a single serum specimen taken at the time of the appearance of the skin eruption may permit a tentative diagnosis of smallpox. The demonstration of a rise in hemagglutination-inhibiting antibodies in several successive daily specimens within the 1st week is conclusive evidence of disease[4] In the unvaccinated patient, complement-fixing antibodies are generally detected after the 10th day, and, in previously vaccinated smallpox patients, after the 6th day.

In general, the clinical improvement of the patient about the 5th day of disease apparently coincides with the rise of neutralizing antibodies in the blood As postulated by Downie, death is likely to occur in the presence of widespread infection of cells and extreme proliferation of virus at the onset of clinical illness and before the appearance of antibodies Severity of disease is determined, therefore, by the severity of invasion up to the 1st or 2d day of illness

C. Pathologic Lesions of Diagnostic Importance

Clinical diagnosis of variola depends largely on the recognition of skin and mucous membrane lesions Aside from blood as a source of material for virus isolation, material obtained from skin and mucous

Laboratory diagnostic procedures include:

1. Microscopic examination of smears taken from scrapings of the base of skin and mucous membrane lesions and stained for the presence of virus elementary bodies
2. Complement fixation tests designed to demonstrate variola antigen in the patient's blood, generally in the pre-eruptive phase, or in the skin lesions
3. Virus culture on the chorioallantoic membrane of embryonated eggs, utilizing blood in the pre-eruptive phase or material from skin lesions
4. Detection of antibody in the patient's serum antibody response to smallpox infection is detected by 3 technics which will be described: (*a*) the hemagglutination-inhibition test, (*b*) the neutralization test, (*c*) the complement fixation test. Demonstration of a 4-fold rise in antibody titer in paired serum specimens drawn 4 to 6 days apart permit definitive serologic diagnosis. A single acute-phase serum may permit a presumptive diagnosis in the unvaccinated patient if antibody titers are moderately elevated

A Collection of Material

Blood Collect 10 ml of venous blood in sterile dry tube with a rubber stopper. Whenever possible, separate serum and clot under aseptic conditions and forward to the laboratory in separate, tightly stoppered, sterile tubes.

Saliva Collect 1 ml in a sterile glass bottle for virus isolation in the maculopapular stage only.

Scrapings and smears Clean the surface of several lesions with a cotton applicator saturated with ether or 70 per cent alcohol

In the maculopapular stage, obtain scrapings from several lesions with a sterile scalpel Make smears on 4 clean glass slides and allow to air-dry.

In the vesicular stage, clean the tops of lesions First aspirate fluid through a 26-gauge needle on a tuberculin syringe or in small capillary tubes with open ends Remove the tops of the lesions and take the scrapings from the base of several lesions. Make smears on 4 glass slides.

In the pustular stage, microscopic examination is of no value because of artifacts produced by degenerating cell particles

Vesicular or pustular fluid Clean lesions, puncture, and aspirate contents with capillary glass pipette. Seal end of capillary in flame and send pipette in stoppered glass tube to laboratory.

As an alternate method of collection, aspirate fluid from vesicles and

affected by the pre-existent eczema They also occur in previously normal areas of skin, which suggests that viremia carries the virus to normal skin sites The mortality rate of eczema vaccinatum, if it occurs in the 1st year of life, is very high

Progressive vaccinia (vaccinia necrosum) is characterized by constant progression over a period of 3 to 5 months of the initial vaccinal lesions, so that adjacent normal skin is destroyed Satellite skin lesions occur in other areas of the skin and bone, as well as in other organs Systemic reaction is initially mild, but death generally occurs after some months unless antibodies can be artificially supplied This condition results from a quantitative defect in gamma globulin (agammaglobulinemia) or a qualitative defect (dysgammaglobulinemia) in which the patient lacks the ability to form specific humoral antibodies against vaccinia[6]

Laboratory determinations of gamma globulin levels and tests for the presence of antibodies against vaccinia are of help in diagnosis and treatment. Virus isolation can be readily accomplished from the lesions and, sometimes, from the blood Laboratory diagnosis of cowpox and vaccinia utilizes the same technics as described for diagnosis of variola.

IV. LABORATORY DIAGNOSTIC PROCEDURES

The selection of laboratory diagnostic procedures depends on the stage of the disease in which material can be collected, as is shown in Table 1.

TABLE 1

LABORATORY TEST IN DIAGNOSIS OF VARIOLA

STAGE OF VARIOLA			DETECTION OF VIRUS			DETECTION OF ANTIBODY					
PRE ERUPTIVE STAGE	CLOTTED BLOOD	SERUM		+							
		CLOT			±						
MACULO-PAPULAR STAGE	CLOTTED BLOOD	SERUM		±		±	±	±			
		CLOT			±						
	SMEARS OF SKIN SCRAPINGS		+		+						
	SALIVA				+						
VESICULAR STAGE	CLOTTED BLOOD	SERUM				+	+	+	±	±	
		CLOT			±						
	SMEAR SCRAPINGS FROM BASE OF VESICLES		+	+	+						
	VESICULAR FLUID		+	+	+						
PUSTULAR STAGE	CLOTTED BLOOD	SERUM				+	+	+	+		±
		CLOT									
	PUSTULAR FLUID		±	+	+						
SCARRING STAGE	CLOTTED BLOOD	SERUM				+	+	+	+	+	+
		CLOT									
	SCABS			+	+						
TIME REQUIRED FOR COMPLETION OF TEST			30 MIN	24 HOURS	3 DAYS	5 HOURS		3 DAYS		24 HOURS	

Modified after A. W Downie and A Macdonald, *Brit. M. Bull* 9·191, 1953.

a. Procedure Fix the air-dried film in methyl alcohol for ½ hour or more. Place the slide in a dry petri dish. Mix equal amounts of solutions of 1 per cent methyl violet in distilled water and 2 per cent sodium carbonate in a test tube Filter immediately onto a slide. Cover the dish with a lid and incubate at 37° C. for 20 to 30 minutes. Rinse in distilled water, air-dry, and examine under the oil immersion lens. Elementary bodies stain light violet

b. Interpretation. Figure 1 shows a smear from the base of a smallpox vesicle and reveals numerous elementary bodies stained by Gutstein's methyl violet method Microscopic examination is most helpful in the papulovesicular phase, but is of little use in the pustular phase because of numerous artifacts Interpretation of microscopic examination requires caution The presence of elementary bodies allows a provisional diagnosis but does not distinguish between variola, vaccinia, or cowpox and virtually rules out the diagnosis of varicella Such a provisional positive report may be most helpful to the clinician, but a negative report does not rule out the presence of variola.

2. *Complement fixation test for detection of antigen in clinical specimens.* Table 1 shows the value of identification of antigen by complement fixation test in various stages of smallpox, using blood, scrapings from macules and papules, vesicular and pustular fluids, and tops of lesions and scabs The detection of variola-vaccinia antigen in the presence of antivaccinia serum does not differentiate between variola and vaccinia, but it conclusively rules out herpes simplex and varicella, which might be considered in the differential diagnosis.

The standard complement fixation test for the variola-vaccinia group of viruses is described in detail on page 358. Demonstration of specific antigen utilizes the same technic with the following modification:

a Reagents. The following antigens can be employed.

Blood Nonhemolyzed serum is inactivated at 56° C. for 30 *minutes* and 2-fold dilutions are made in normal saline.

Smears and scrapings of macules and papules. Duplicate of smears originally prepared for microscopic examination (see p 348) provide useful sources of antigen for identification by complement fixation test The technic is as follows:

Place 1 or 2 smears in a sterile petri dish containing 1.0 ml. c solution Scrape the slide with a sterile scalpel, Wash the sl

pustules, using a 1/4 ml. tuberculin syringe and a 26-gauge needle. Return the syringe and contents intact to the laboratory in a rubber stopper-sealed glass tube.

Tops of lesions and scabs. Collect the tops of lesions or scabs in a sterile tube with rubber stopper.

1. *Packing and shipping of specimens.* Place in metal containers with screw top. Carefully label "HIGHLY INFECTIOUS MATERIAL, SUSPICIOUS OF SMALLPOX" so that proper precautions will be taken before the package is opened in the laboratory. A history sheet should accompany the specimen, giving the following information.

1 Name and age of patient
2. Date and place of collection of specimen
3. Date of onset of illness
4. Date of last vaccination
5. Known exposure to smallpox or vaccinia
6. Known exposure to chickenpox and/or history of known previous chickenpox.

Although refrigeration is desirable for specimens which are submitted, virus isolation can often be accomplished without it. Refrigeration is not required for microscopic examination, for the demonstration of antigen, or for serologic testing Blood specimens should be frozen only after serum and clot have been separated

2 *Precautions for handling of infectious material.* All laboratory workers should be vaccinated yearly and revaccinated promptly whenever variolous material is introduced into the laboratory. It must be noted that slides submitted for microscopic examination can, of course, be a source of laboratory infections.

B ISOLATION AND IDENTIFICATION OF THE VIRUS

1. *Microscopic examination of smears.* Only 1 or 2 of several available smears should be stained for identification of elementary bodies, because smears are also a valuable source of variola antigen for identification by complement fixation test A number of suitable stains for demonstration of elementary bodies may be employed, but Gutstein's methyl violet stain has been found to be the most simple, satisfactory technic [7] The solutions required are 1 per cent methyl violet in distilled water and 2 per cent sodium carbonate.

a. Procedure. Fix the air-dried film in methyl alcohol for ½ hour or more. Place the slide in a dry petri dish Mix equal amounts of solutions of 1 per cent methyl violet in distilled water and 2 per cent sodium carbonate in a test tube Filter immediately onto a slide Cover the dish with a lid and incubate at 37° C. for 20 to 30 minutes Rinse in distilled water, air-dry, and examine under the oil immersion lens Elementary bodies stain light violet

b Interpretation. Figure 1 shows a smear from the base of a smallpox vesicle and reveals numerous elementary bodies stained by Gutstein's methyl violet method Microscopic examination is most helpful in the papulovesicular phase, but is of little use in the pustular phase because of numerous artifacts Interpretation of microscopic examination requires caution The presence of elementary bodies allows a provisional diagnosis but does not distinguish between variola, vaccinia, or cowpox and virtually rules out the diagnosis of varicella. Such a provisional positive report may be most helpful to the clinician, but a negative report does not rule out the presence of variola.

2 *Complement fixation test for detection of antigen in clinical specimens.* Table 1 shows the value of identification of antigen by complement fixation test in various stages of smallpox, using blood, scrapings from macules and papules, vesicular and pustular fluids, and tops of lesions and scabs The detection of variola-vaccinia antigen *in the presence* of antivaccinia serum does not differentiate between variola and vaccinia, but it conclusively rules out herpes simplex and varicella, which might be considered in the differential diagnosis

The standard complement fixation test for th [illegible]

a Reagents The following antigens can be employed.

Blood Nonhemolyzed serum is inactivated at 56° C *for 30 minutes* and 2-fold dilutions are made in normal saline.

Smears and scrapings of macules and papules. Duplicate of smears originally prepared for microscopic examination (see p 348) provide useful sources of antigen for identification by complement fixation test. The technic is as follows:

Place 1 or 2 smears in a sterile petri dish containing 1.0 ml of saline solution. Scrape the slide with a sterile scalpel. Wash the slide down

from time to time and leave at 4° C. for 1 hour. Remove 0 2 ml of the saline solution and dilute with equal parts of saline containing antibiotics (penicillin, 1,000 U/ml and streptomycin, 5 mg /ml.) for virus isolation purposes (see p 352). To the 0.8 ml. remaining in the petri dish add 0.7 ml. of saline; this material is used in tests for the presence of complement-fixation antigen.

Vesicular and pustular fluid. If collected in capillary tubes, crush these in test tube by grinding with glass rod. If material is submitted in a tuberculin syringe, wash needle and syringe with 0.5 ml of saline solution back and forth in the original storage tube. Add 2 ml. of ether for 60 minutes Remove ether by pipette and gentle warming in a warm water bath. Add physiologic saline to a final volume of 1.7 ml. Remove 0.2 ml for virus isolation purposes (see p 352). Use remaining 1 5 ml. for complement fixation test

Vesicle, pustule, and scab tissue. Remove small portion of material for egg inoculation and suspend in normal saline solution containing antibiotics. Dry rest of material in a phosphorus pentoxide desiccator (or calcium chloride) for several hours and weigh. Subsequent steps are those described by MacCallum.[8] Grind scabs in an enclosed tube (Ten Broeck grinder), add several ml. of ether and allow to stand for about 30 minutes. Remove ether by pipette and gentle warming in warm water bath and perform final grinding. Add 10 times the weight by volume of 9 per cent sodium chloride Mix with the powdered material and place in refrigerator at 4° C for 2 hours Add distilled water to 10 times the volume of the saline solution to a final 1:100 dilution of material in normal saline Centrifuge suspension at 2,000 r p m for 10 minutes. Remove supernatant fluid and heat at 58° C. for 30 minutes. Use this as antigen (A minimal volume of 1.4 ml is required for the test.)

Immune serum. Prepare variola-vaccinia immune serum as follows: Scarify the skin on a rabbit at 5 or 6 sites and inoculate with calf lymph vaccine Ten days after lesions have healed give 3 intravenous injections, at weekly intervals, of a saline suspension of vaccinia elementary bodies prepared as outlined on page 356. Bleed rabbit 2 weeks after the last injection.

To determine the optimal dilution of immune serum to be used in the complement fixation test a box titration, using known antigen, is done as described below. The dilution of serum giving 4+ or 3+ with the highest reaching dilution of antigen is used in the actual test. For exam-

ple: from the results in the box shown below, a serum dilution of 1:40 would be employed.

ANTIGEN DILUTION

		10	20	40	80	160	320
Serum Dilution	20	4+	4+	4+	4+	2+	0
	40	4+	4+	4+	4+	0	0
	80	4+	4+	4+	2+	0	0
	160	4+	4+	0	0	0	0
	320	4+	3+	0	0	0	0
	640	0	0	0	0	0	0

b The test. The test proper is set up as shown in Table 2.

TABLE 2

	Vaccinia Immune Serum	Normal Serum	Unknown Antigen	Saline	Complement
Tube 1 (test)	0 2	—	0 2	—	0 20
Tube 2 (N S)*	—	0 2	0 2	—	0.20
Tube 3 (A C)*	—	—	0 2	0 20	0 20
Complement control	—	—	0 2	0 35	0 05
Complement control	—	—	0 2	0 30	0.10
Complement control	—	—	0 2	0 25	0 15
Complement control	—	—	0 2	0 20	0 20
Normal serum control	—	0 2	—	0.20	0 20
Hemolytic control	—	—	—	0 40	0 20
Cell control	—	—	—	0.60	—

* N S —Antigen nonspecific control
A C —Antigen anticomplement control

The 4-tube complement control is necessary in order to ensure that the dilution contains exactly 2 units in 0 2 ml. The 1st tube containing 0 05 ml. of complement (less than 1 unit) should show only partial hemolysis, and the other 3 tubes containing 1 unit or more of complement should show complete hemolysis

Incubate at 4° to 6° C. overnight, followed by 10 minutes in 37° C water bath For more rapid testing, 4-hour incubation may sometimes suffice, but is not recommended Add sensitized sheep cells and shake. Incubate in 37° C water bath for 30 minutes Read

c Interpretation. The unknown antigen is identified as variola-vaccinia if tube 1 (test) is read as 4+ and tube 2 (N S.) and tube 3 (A.C.) are read as 0 (complete hemolysis). The hemolytic control should show complete hemolysis and the cell control should show no hemolysis.

3 *Isolation of virus in fertile hens' eggs*

a Preparation of material for inoculation.

Blood Heparinized blood may be employed, but it is well to separate cells from serum because the presence of neutralizing antibodies in the serum may mask infective virus in the cells. Inoculum may consist of a suspension of cells in saline solution When heparinized blood is available a heavy suspension of buffy coat containing a predominance of white blood cells is preferred

Vesicular and pustular fluid. Dilute whatever amount is available to 1 0 ml. in saline solution containing 1,000 U/ml. of penicillin and 5 mg /ml. of streptomycin. Inoculate 0 2 ml on the chorioallantoic membrane of each of 4 eggs Inoculate 0 2 ml. on a blood agar plate and incubate for 24 hours to test for presence of bacteriologic contamination

Tops of lesions and scabs Dilute suspension of carefully ground lesion material to 1 ml. in saline solution containing penicillin (1,000 U/ml.) and streptomycin (5 mg /ml) Inoculate 0 2 ml. into each of 4 eggs, as with vesicular and pustular fluid.

b. Method of egg culture. Candle 4 or more 10- to 13-day embryonated hens' eggs Mark a spot between blood vessels for the respective windows. Paint this area of the shell with tincture of iodine Carefully make a small perforation over the previously outlined air sac. Remove a small area of the shell over the chorioallantois and place a drop of sterile saline solution on the shell membrane. Gently "tease" apart the shell membrane and let the drop of saline solution spread between the shell membrane and the chorioallantois, which will drop away. Gentle suction through the air sac will facilitate this. Drop 0 2 ml of inoculum on the chorioallantois with a tuberculin syringe with a 23-gauge needle. Gently rotate the egg to permit the inoculum to spread over the entire chorioallantoic membrane. Seal the holes with cellophane tape and incubate in a humid atmosphere at 37° C for 72 hours Remove the

round

ariola,

vaccinia, and herpes simplex.

c. Interpretation Variola produces fairly small, discrete, circular plaques which are dome-shaped and show no surrounding tissue destruction or hemorrhage (Fig 2). Vaccinia virus causes lesions which are flat-

ter, with considerable necrosis and hemorrhage (Fig. 3). Herpes simplex lesions, on primary isolation of the virus, are usually very small, tend to be oval, with no evidence of necrosis and little tissue reaction (Fig 4).

An inoculum obtained from varicella will produce no lesions. Lesions indistinguishable from those caused by variola major are produced by variola minor (alastrim)

4. *Identification of virus in infected membrane suspension.* Final identification is obtained as follows.

Prepare a 20 per cent saline solution suspension of the harvested membranes, centrifuge at 1,500 r p m for 10 minutes, and use *(a)* as a hemagglutinating antigen for susceptible chicken cell suspension, *(b)* as a complement-fixing antigen in the presence of antivaccinia serum, *(c)* as inoculum for intradermal inoculation of rabbits, and *(d)* as inoculum for second chorioallantoic passage

a Hemagglutination-inhibition test Proceed as described for the hemagglutination-inhibition test (p 354) for the variola-vaccinia group. Substitute the unknown chorioallantois in the antigen titration for the 20 per cent known virus suspension A known vaccinia or variola suspension is tested in a similar manner Specific hemagglutination will distinguish the variola-vaccinia group from herpes simplex.

b Complement fixation test The chorioallantoic suspension is used as an antigen in the presence of antivaccinia serum, as described (p 349) This test will not distinguish variola from vaccinia but will differentiate that group of viruses from herpes simplex

c Rabbit skin inoculation Inoculation of rabbit skin is a confirmatory technic for differentiation between variola and vaccinia isolated on the chorioallantoic membrane, after the unknown virus is found to belong to the variola-vaccinia group Intradermal or scratch inoculation with a 10^{-3} suspension of chorioallantois containing vaccinia virus will produce necrotic lesions within 3 days on the shorn rabbit's back. Some variola strains produce lesions in the rabbit skin on 1st passage, but lose that ability on 2d rabbit passage On the other hand, vaccinia retains its pathogenicity for rabbit skin for an indefinite period. Failure to produce a lesion on 1st passage in the rabbit [illegible]

[illegible]

complement fixation tests. Conclusive *identification is obtained* by passage of a suspension of the excised rabbit skin on the 3d day after inoculation to another rabbit by similar technic.

c Interpretation. The unknown antigen is identified as variola-vaccinia if tube 1 (test) is read as 4+ and tube 2 (N.S.) and tube 3 (A C.) are read as 0 (complete hemolysis). The hemolytic control should show complete hemolysis and the cell control should show no hemolysis.

3 *Isolation of virus in fertile hens' eggs.*

a Preparation of material for inoculation.

Blood Heparinized blood may be employed, but it is well to separate cells from serum because the presence of neutralizing antibodies in the serum may mask infective virus in the cells. Inoculum may consist of a suspension of cells in saline solution When heparinized blood is available a heavy suspension of buffy coat containing a predominance of white blood cells is preferred.

Vesicular and pustular fluid. Dilute whatever amount is available to 1 0 ml in saline solution containing 1,000 U/ml of penicillin and 5 mg /ml of streptomycin Inoculate 0 2 ml on the chorioallantoic membrane of each of 4 eggs Inoculate 0 2 ml on a blood agar plate and incubate for 24 hours to test for presence of bacteriologic contamination

Tops of lesions and scabs Dilute suspension of carefully ground lesion material to 1 ml. in saline solution containing penicillin (1,000 U/ml.) and streptomycin (5 mg /ml) Inoculate 0.2 ml. into each of 4 eggs, as with vesicular and pustular fluid

b. Method of egg culture Candle 4 or more 10- to 13-day embryonated hens' eggs Mark a spot between blood vessels for the respective windows Paint this area of the shell with tincture of iodine. Carefully make a small perforation over the previously outlined air sac Remove a small area of the shell over the chorioallantois and place a drop of sterile saline solution on the shell membrane. Gently "tease" apart the shell membrane and let the drop of saline solution spread between the shell membrane and the chorioallantois, which will drop away. Gentle suction through the air sac will facilitate this Drop 0 2 ml. of inoculum on the chorioallantois with a tuberculin syringe with a 23-gauge needle Gently rotate the egg to permit the inoculum to spread over the entire chorioallantoic membrane. Seal the holes with cellophane tape and incubate the egg in a humid atmosphere at 37° C for 72 hours Remove the chorioallantois and float it in saline solution against a black background to permit a macroscopic differentiation between the lesions of variola, vaccinia, and herpes simplex.

c Interpretation Variola produces fairly small, discrete, circular

b. Test procedure.

Antigen titration Set up series of 10 tubes (10 × 75 mm.) Add 0.5 ml of 0 85 per cent saline solution to all the tubes Add 0 5 ml of 20 per cent virus stock suspension to the 1st tube, using 1 ml. serologic pipette. Mix the contents of the 1st tube, using new pipette. Transfer 0 5 ml of the mixture to the 2d tube (1:20) Two-fold dilutions are made similarly up to 1.1,024. Add 0 25 ml of 0.5 per cent chicken cell suspension to all the tubes and a control tube containing 0.5 ml of saline solution Shake the rack to mix and allow to stand for about an hour at room temperature until the cells have settled (A titer of 1:640 or 1 1,280 is desirable for suitable antigen)

One hemagglutinating unit is defined as the least amount of antigen in 0 25 ml, which produces definite agglutination. A *positive reaction* is indicated by a homogeneous shield of agglutinated cells over the bottom of the tube, a *negative reaction* is shown by a button of settled cells at the bottom of the tube Two units are used for the test

The test The test is based on 2-fold serial dilutions of serum in 0.25 ml of volume, to which 2 hemagglutinating units of antigen in 0 25 ml of chicken cell suspension are added The serum titer is the greatest dilution which completely inhibits hemagglutination

Two-fold dilutions of test sera, 0 25 ml, per dilution, are set up in dilutions from 1:5 to 1 2,560—by adding 0 1 ml of serum to 0 4 ml of saline solution in the 1st tube, mixing and carrying 0 25 ml over into 0 25 ml, saline solution in the 2d tube and subsequent tubes to complete a series of 10 tubes Set up positive and negative serum controls—the positive serum to measure the sensitivity of the test, the negative to assure specificity and eliminate a nonspecific lipid agglutination To each tube, add 0 25 ml of virus suspension containing 2 hemagglutinating units. For example if the end point is 1 1,024, 2 units are represented by 1 512 dilution

A virus control is set up in the following manner Use a series of 6 tubes. Add 0 5 ml of saline solution to the tubes, except the 1st tube Add 0 5 ml. of the diluted antigen (containing 2 units in 0 25 ml) to the 1st and 2d tube Mix the contents of the 2d tube and transfer to the 3d tube. Two-fold dilutions are made similarly up to the 6th tube Shake all racks and place in incubator at 35° to 37° C for 1 hour Add 0 25 ml of the 0 5 per cent chicken cell suspension to all tubes. Shake well and allow to stand at room temperature until the cells have settled (about 1 hour). Add cells to a tube containing 0.5 ml saline solution to serve as a

C Serologic and Immunologic Methods of Diagnosis

A significant rise in antibodies against the variola-vaccinia group of viruses is of diagnostic importance. The detection of hemagglutination-inhibiting antibodies or complement-fixing antibodies in patients who have not been vaccinated in the previous 24 months, or a 4-fold rise in antibodies in 2 successive samples of serum taken 4 to 6 days apart from patients more recently vaccinated, can materially aid in specific early diagnosis Tentative diagnosis can be made in the unvaccinated individual by the demonstration of hemagglutinating or neutralizing antibodies in a single specimen of serum taken shortly after the appearance of the rash

1. *Hemagglutination-inhibition test*

a. Reagents.

Virus antigen suspension Prepare antigen by inoculating the dropped chorioallantoic membranes of 11- to 13-day embryonated hens' eggs with 0 2 ml. of a 1 100 to 1·1,000 dilution of egg-adapted vaccinia virus or calf lymph Incubate 3 days at 37° C. Harvest, pool, and grind in a mortar the chorioallantoic membranes of all eggs, dead or alive. Dilute to 20 per cent suspension by weight in physiologic saline. After 1,500 r.p m centrifugation for 10 minutes, remove the supernatant fluid as stock virus suspension This remains stable indefinitely in the frozen state, and as long as 3 months at 4° C

Chicken cell suspension Only red blood cells of certain adult chickens are agglutinable by the variola-vaccinia virus Select appropriate donors by testing with 0.5 ml of known virus suspension or with 0 5 ml. of cardiolipin microflocculation antigen in dilution of 1.10,000 Chicken cells agglutinated by at least 1 32 dilution of 20 per cent C.A.M. suspension (0.25 ml of 0 5 per cent cell suspension plus 0 5 ml of virus suspension) are considered appropriate.

Blood is taken in Alsever's solution in a proportion of approximately 1:5. (Chicken cells in Alsever's solution are stored at 4° C. and are usable for about 2 weeks.) Before use, remove an appropriate amount, wash 3 times in 0 85 per cent saline solution. Pack by centrifugation at 1,800 r.p m. for 10 minutes, and dilute to 0.5 per cent suspension in 0 85 per cent saline solution

Sera. Dilute sera to 1 5 with 0 85 per cent saline solution and inactivate at 60° C. for 30 minutes. Use hyperimmune rabbit serum as a positive control, and normal rabbit serum for negative control. (The negativity should be demonstrated by test)

Incubate dilutions in the water bath at 37° C. for 30 minutes At least 5 eggs per dilution are inoculated on the chorioallantoic membrane with 0.2 ml. of inoculum per egg After 48 hours' incubation at 37° C, harvest chorioallantoic membranes and place in petri dishes with salt solution Count pocks on a bacterial colony counter, with an average pock count obtained from 5 eggs Use a dilution which produces about 100 to 500 pocks for the test.

Sera Inactivate sera (dilute with sterile saline solution if necessary) in water bath at 56° C. for 30 minutes

Eggs Use 10- to 13-day-old embryonated hens' eggs, Preparation of dropped chorioallantoic membrane is described on page 352

b The test Use inactivated serum undiluted and diluted 1 2. Place serum (0 1 ml per egg) in a sterile tube with a cork or a rubber stopper Make virus dilutions double strength so that after addition of equal amounts of sera, they are of desired dilution

After inactivation, mix equal amounts of serum and virus dilution and incubate in water bath at 37° C, or at room temperature, for 30 minutes For very low titered sera, 4 hours' incubation at room temperature is advisable Two controls are set up. (*a*) virus-saline mixture (equal amounts of each) serving as a standard in pock counting, and (*b*) virus-vaccinia immune rabbit serum mixture as a positive control After 30 minutes' incubation, place the tubes with inoculum in an ice water bath or in an icebox until inoculated Inoculate 0 2 ml of the inoculum on a dropped chorioallantoic membrane, using a 1 ml tuberculin syringe and a 23-gauge needle Gently rotate the egg to let the inoculum spread over the chorioallantoic membrane Seal the holes with cellophane tape.

Harvest chorioallantoic membranes after a 48-hour incubation at 37° C Obtain an average number of pocks for each dilution Obtain percentage of pock reduction from the number of pocks of virus-sera mixture and that of the control virus-saline mixture A 50 per cent reduction in pock count is considered significant If 50 per cent or greater pock reduction has occurred with undiluted serum, titration may be done on a step-wise fashion by using serum dilutions of 1:2, 1:4, and 1 8 and note results If neutralization again occurs, dilutions of 1 16 and 1:32 are tested Or a wider range may be employed after the preliminary test Titer is the highest dilution of serum which permits an average pock reduction of 50 per cent or greater

control of the cell suspension. Read test after the cells are sedimented This is facilitated by holding the racks over a mirror if 13/32-inch diameter holes have been bored through the bottom of the racks, or if wire racks are used

The negative serum control should show hemagglutination throughout. The positive serum control should not differ more than 1 tube from the previously determined titer. The virus control should show agglutination in the first 3 tubes.

The test serum titers are recorded as the highest serum dilution giving complete inhibition of agglutination (a pattern of a sharply demarcated button of red cells similar to the cell control).

c. Interpretation. A 4-fold rise in titer of paired serum specimens is diagnostic of infection with an agent of the variola-vaccinia group A single serum specimen drawn on the 3d to 5th day of the illness may strongly suggest the diagnosis if a titer of 1:160 or above is obtained in a person not recently vaccinated

2. *Neutralization test*

a Reagents A 20 per cent saline suspension of chorioallantoic membrane infected with vaccinia virus may be used Because variola produces smaller, more discrete pocks it is often preferred as an antigen for egg neutralization tests.

Purified suspensions More clear-cut results are obtained with partially purified elementary body suspensions than with crude suspension Purified suspensions are made as follows: prepare a 20 per cent saline suspension of chorioallantoic membranes infected with variola or vaccinia virus Shake this suspension in a tightly stoppered flask with glass beads, using 9 times its volume of sterile, buffered distilled water as diluent. Centrifuge at low speed for 10 minutes Draw off supernatant fluid and centrifuge in an angle head at either 5,000 r p m. for 2 hours or at 13,000 r p m for 20 minutes. Resuspend the sediment in saline to ⅕ the original volume. The suspension is cultured for sterility and may be kept frozen in small lots for repeated use

Virus titration. Set up a series of tubes containing 0 5 ml. sterile saline solution in the 1st tube and 4.5 ml. of saline in the balance of the tubes Add 0 5 ml of 20 per cent chorioallantoic membrane or elementary body suspension to the 1st tube; transfer 0 5 ml. of the mixture to the 2d tube, transfer 0 5 ml. from the 2d mixture to the 3d tube, and so on. For titration use 5 dilutions, which will bracket expected end point

Incubate dilutions in the water bath at 37° C. for 30 minutes At least 5 eggs per dilution are inoculated on the chorioallantoic membrane with 0 2 ml of inoculum per egg After 48 hours' incubation at 37° C, harvest chorioallantoic membranes and place in petri dishes with salt solution *Count pocks on a bacterial colony counter, with an average pock count* obtained from 5 eggs Use a dilution which produces about 100 to 500 pocks for the test

Sera. Inactivate sera (dilute with sterile saline solution if necessary) in water bath at 56° C for 30 minutes

Eggs Use 10- to 13-day-old embryonated hens' eggs Preparation of dropped chorioallantoic membrane is described on page 352

b The test Use inactivated serum undiluted and diluted 1 2 Place serum (0 1 ml per egg) in a sterile tube with a cork or a rubber stopper Make virus dilutions double strength so that after addition of equal amounts of sera, they are of desired dilution

After inactivation, mix equal amounts of serum and virus dilution and incubate in water bath at 37° C, or at room temperature, for 30 minutes For very low titered sera, 4 hours' incubation at room temperature is advisable Two controls are set up (*a*) virus-saline mixture (equal amounts of each) serving as a standard in pock counting, and (*b*) virus-vaccinia immune rabbit serum mixture as a positive control After 30 minutes' incubation, place the tubes with inoculum in an ice water bath or in an icebox until inoculated Inoculate 0 2 ml of the inoculum on a dropped chorioallantoic membrane, using a 1 ml. tuberculin syringe and a 23-gauge needle Gently rotate the egg to let the inoculum spread over the chorioallantoic membrane Seal the holes with cellophane tape.

Harvest chorioallantoic membranes after a 48-hour incubation at 37° C Obtain an average number of pocks for each dilution. Obtain percentage of pock reduction from the number of pocks of virus-sera mixture and that of the control virus-saline mixture A 50 per cent reduction in pock count is considered significant If 50 per cent or greater pock reduction has occurred with undiluted serum, titration may be done on a step-wise fashion by using serum dilutions of 1 2, 1 4, and 1 8 and note results If neutralization again occurs, dilutions of 1 16 and 1 32 are tested. Or a wider range may be employed after the preliminary test Titer is the highest dilution of serum which permits an average pock reduction of 50 per cent or greater

c. Interpretation. The presence of any neutralizing antibodies as judged by 50 per cent pock reduction may be significant. Titers of 1:8 or 1:16 usually can be expected after primary vaccination and are usually higher (1.32 or 1.64) in variola major.

3. *Complement fixation test.*

a. Reagents. An 0 85 per cent saline solution containing 1 0 ml of 10 per cent Mg SO_4 per liter (Kolmer saline) may be used.

Sensitized cell suspension. Collect sheep cells in Alsever's solution (equal parts). Wash 3 times at 1,500 r.p m in saline for 8 minutes After last washing pack at 2,000 r p m for 15 minutes and use only if supernatant fluid is colorless and crystal clear. Make 2.5 per cent cell suspension in saline solution

Amboceptor (hemolysin). Two units are contained in 0 2 ml Preparation of stock solution of 1:100: 98 ml of Kolmer saline, 2 ml. of amboceptor preserved in 50 per cent glycerin. Store in refrigerator.

Titration Prepare a 1.1,000 solution of amboceptor (0 3 ml of stock 1 100 dilution plus 2.7 ml of Kolmer saline).

PREPARATION OF DILUTION

0 5 ml amboceptor (1 1,000)	1.1,000
0 5 ml amboceptor (1:1,000) + 0 5 ml saline	1·2,000
0 5 ml amboceptor (1'1,000) + 1 0 ml saline	1 3,000
0.5 ml amboceptor (1:1,000) + 1 5 ml saline	1·4,000
0.5 ml amboceptor (1 1,000) + 2 0 ml saline	1:5,000
0 5 ml amboceptor (1 3,000) + 0 5 ml saline	1 6,000
0 5 ml amboceptor (1 4,000) + 0 5 ml saline	1 8,000
0 5 ml amboceptor (1 5,000) + 0 5 ml saline	1 10,000
0 5 ml amboceptor (1 6,000) + 0 5 ml saline	1·12,000
0 5 ml amboceptor (1 8,000) + 0 5 ml saline	1·16,000

TITRATION

0 2 ml amboceptor dilutions (1 1,000 to 1 16,000)
0 2 ml 1 30 complement (0 1 ml comp. + 2 9 ml saline)
0 4 ml Kolmer saline
0 2 ml of 2 5 per cent sheep cells (suspended in Kolmer saline from the previously washed and packed cells)

Shake to mix, then incubate in a 37° C. water bath for 30 minutes and read. The highest dilution of amboceptor which shows complete hemolysis represents ONE UNIT Two units are used in the test, for example: If the 1:10,000 dilution shows complete hemolysis, 2 units would be contained in 0 2 ml of a 1:5,000 dilution of amboceptor. Rapidly mix

equal parts of the 2.5 per cent cell suspension and amboceptor dilution (as determined by titration), by pouring back and forth several times These *must* stand at room temperature for 10 minutes before being used.

Complement. Titrate 2 exact units to be contained in 0.2 ml Dilute antigen as to be used in the test

Titration Dilute lyophilized complement to 1:30 (0.1 ml comp. + 2.9 ml of saline) Titrate in the following amounts·

Tube	1	2	3	4	5	6	7	8	9
Comp (1:30)	0.15	0.13	0.12	0.11	0.10	0.09	0.08	0.07	0.06
Antigen	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Saline	0.25	0.27	0.28	0.29	0.30	0.31	0.32	0.33	0.34

Incubate in 37° C water bath for 30 minutes, then add 0.40 ml sensitized cells to all tubes Shake to mix and incubate in a 37° C water bath for 30 minutes Read. Two exact units are used in the test—the tube which contains the least amount of complement showing complete hemolysis represents *1 exact unit*. The complement may be diluted to contain 2 exact units in 0.2 ml, by using the following formula: 2V · 30 as 0.2 . X. Where V represents the volume of complement used in the last tube showing complete hemolysis in the complement titration, X represents dilution of complement to be used in the test.

Antigen. Two units are contained in 0.2 ml

Use supernate of high speed centrifugation of 20 per cent chorio-allantoic membrane suspension infected with calf lymph vaccinia virus This soluble antigen will keep under refrigeration for 1 to 2 years and can be formalinized Control antigen is prepared from noninfected membranes in a similar manner. Minced infected rabbit skin can also serve as a source of high titer antigen.

Titration is conducted by testing serial 2-fold dilutions of antigen

Box titration Dilute immune serum serially, starting at a dilution which is 4-fold below the expected titer of the serum, and carry through a dilution which is 4-fold greater than the expected titer of the serum Dilute the antigen serially, starting at 1:2 and continue through 1:128. These dilutions may be altered if previous experience indicates that a

different range may be more satisfactory. Add 0.2 ml. of 2 exact units of dilute complement, as determined by the complement titration and calculation. Include controls on all reagents.

Set up box titration in the following manner. duplicate rows of 0.2 ml of serum dilutions, 0 2 ml. of antigen dilutions 1:2 through 1:128 to each respective serum dilution. 0.2 ml of complement diluted to contain 2 exact units in 0.2 ml. to each tube. Shake racks well and allow to stand in refrigerator overnight Follow with 10 minutes in 37° C. water bath to warm Add 0 4 ml of sensitized sheep cells to each tube, place in 37° C. water bath for 30 minutes, and then read

One unit is determined by the titration as the highest dilution of antigen giving a 3+ or 4+ reaction with the highest dilution of immune serum The dilution of antigen to employ in the main test is one calculated to give 2 units in 0.2 ml. If, for example, 1 unit was found in 0 2 ml of the 1.16 dilution of the antigen, the antigen would be diluted 1:8 for use in the test

Sera Inactivate in initial (1·4) dilution for 30 minutes in a 60° C water bath. Two-fold serial dilutions are made in Kolmer saline, usually beginning with a 1 4 dilution

b The test. The test proper is set up as indicated in the following chart

	Serum Dilution	Specified Antigen 2 units	Non-specific Antigen	Saline	Complement 2 units
Test proper (with specific antigen)	0 2	0 2	—	—	0 20
Test serum-nonspecific control	0 2	—	0.2	—	0 20
Test serum A C control	0 2	—	—	0 20	0 20
Complement control and specific	—	0.2	—	0.35	0.05
antigen control	—	0 2	—	0.30	0 10
	—	0 2	—	0 25	0 15
	—	0 2	—	0 20	0.20
Nonspecific antigen control	—	—	0 2	0 20	0 20
Hemolytic control	—	—	—	0.40	0 20
Sheep cell control	—	—	—	0 60	—

Shake to mix. Incubate overnight at 4° to 6° C Follow with 10 minutes in 37° C. water bath Add 0.4 ml of sensitized cells (prepared 10 minutes previously) to all tubes. Place tubes in 37° C. water bath for 30 minutes or less, as determined by 4-tube complement control Remove when 1 unit clears. Allow the tubes to stand about 15 minutes and read.

Reading of the test:

4+	0 hemolysis
3+	25 per cent hemolysis
2+	50 per cent hemolysis
1+	75 per cent hemolysis
±	90 per cent hemolysis
0	100 per cent hemolysis

c. Interpretation After the 6th day of illness, variola patients who have been previously vaccinated can be expected to have complement-fixing antibody titers of 1 16 or higher Unvaccinated patients do not develop such titers until after the 10th day of the disease

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V REFERENCES

1 Fenner, F Mouse-pox (Infectious Ectromelia of Mice) A Review. *J Immunol*, 63 341–373, 1949

2 [illegible]

3 [illegible]

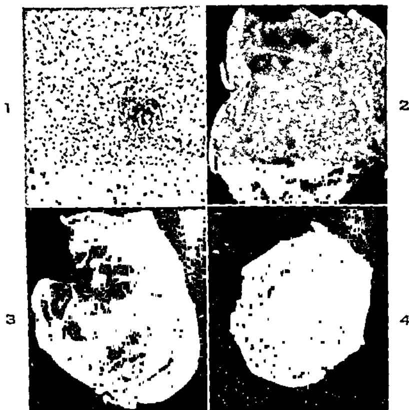
4 [illegible] inen bei Variolapatienten als diagnostisches Hilfsmittel *Ztschr Hyg*, 131 555–567, 1950

5. Kempe, C H Faulty Immune Mechanisms in Serious Complications of Smallpox Vaccination *Fed Proc* 14 468, 1955

6 Barbero, G J, Gray, A, Scott, T F McN, and Kempe, C H Vaccinia Gangrenosa Treated with Hyperimmune Vaccinal Gamma Globulin *Pediatrics*, 16 609–618, 1955

7 Gutstein, M New Direct Staining Methods for Elementary Bodies *J Path and Bact*, 45 313–314, 1937,

8 MacCallum, F O Early Diagnosis of Smallpox Chapter 24 in *The Dynamics of Virus and Rickettsial Infections* New York The Blakiston Company, Inc, 1954, pp 324–333



* Fig 1 Variola elementary bodies—Smears from skin lesions

Fig 2 Variola—Chorioallantois, 3 days after inoculation

Fig 3 Vaccinia—Chorioallantois, 3 days after inoculation

Fig 4 Herpes simplex—Chorioallantois, 3 days after inoculation

* Gutstein stain × 1400 Courtesy of A W Downie

YELLOW FEVER

JOHN C. BUGHER, M.D.
Director for Medical Education and Public Health
The Rockefeller Foundation, New York City

THE clinical manifestations in yellow fever are so variable that positive diagnosis is impossible without laboratory assistance. In only a minority of cases is the classical clinical picture encountered. While there are numerous laboratory procedures of value, only those will be considered here which lead to the establishment of the diagnosis; those having presumptive value only will be omitted

I. ISOLATION AND IDENTIFICATION OF THE VIRUS

For the certain diagnosis of yellow fever, three lines of investigation are available:

1. Isolation of yellow fever virus from patient's blood.
2. Demonstration of specific histopathologic lesions at autopsy.
3. Demonstration of a marked rise in specific antibody content of the blood serum during convalescence.

A. Isolation of Virus from Patient's Blood

During the period of circulation of active virus in the patient's blood, the presence of the infective agent may be demonstrated by inoculating whole blood or serum into a susceptible animal. The test animals most commonly used are the rhesus monkey and the white mouse Many monkeys native to South America and Africa may also be employed satisfactorily if due consideration is paid to the variability in susceptibility of these animals and to the tendency for most of the species to have the disease in a very mild form.

Inoculation of the monkey may be by the subcutaneous or intraperitoneal routes. On the other hand, the adult white mouse is relatively insusceptible by these routes so that the suspected serum must be inoculated directly into the brain

The prospects of successful demonstration of virus are excellent during the first 3 days of the disease, irrespective of the degree of illness; only rarely can virus be shown after the 6th day after the clinical onset. There is a marked variability not only in the time clinical manifestations of yellow fever but also in the time at which demonstrable virus may appear in the blood stream as well as in the length of time it circulates and the amount present as judged by *titration in mice*. In a suspected case, therefore, it is wise to make daily bleedings for 5 days, ... as possible in the course of the disease. It is desirable ... e sufficient

1. *Monkeys.* When rhesus monkeys are employed as test animals, the inoculum should be 1 to 2 ml. of the suspected serum The blood of the animal to be used is examined before inoculation of the virus to make sure that it is free of yellow fever antibodies A sample of this pre-experimental serum should be preserved for subsequent comparison. The rectal temperature of the test animal is taken morning and evening, and elevations above 40° C. can be considered as significant In such event the animal should be bled (preferably from the leg vein) and the serum subinoculated into a group of mice (conventionally 6) If the case is one of exceptional importance, then such bleedings may be performed daily, irrespective of evidences of fever The serum remaining from each bleeding may, if desired, be preserved by freezing and drying or in a low temperature cabinet In the event that the monkey should suffer a fatal illness, autopsy with special attention to the microscopic findings in the liver may confirm the diagnosis at once without waiting for the final evidence in the mice The most satisfactory identification of the virus, however, is in the mouse passage material When the mice become ill, the brains of 1 or 2 whose symptoms are typical may be triturated in 3.0 ml each of 10 per cent normal monkey or human serum in saline diluent Further dilutions of this virus preparation are allowed to react with normal and yellow fever immune sera and the mixtures are tested for surviving virus by inoculation into mice The technic is that of the standard intracerebral protection test Since it is essential that the neutralization test be specific, it is recommended that the normal and the immune sera be from the same monkey—taken before and after known yellow fever immunization—so that differences other than the presence of yellow fever antibodies will be eliminated.

2 *Mice.* Since mice are presumed to be available, it is recommended that a group of these animals be inoculated with each original blood specimen Owing to the high cost of rhesus monkeys, it is usually not possible to devote several of them to a single patient, and where multiple bleedings are obtained in the acute stage of illness, they must of necessity be given to the same animal The simultaneous inoculation of mice permits more exact information to be obtained with regard to the days of virus circulation

When monkeys are not available, mice can be used alone with good prospect of success But different races of white mice vary markedly in their susceptibility to yellow fever virus, and strains of the virus differ in pathogenicity for mice. These facts have led various workers to mis-

trust the use of mice *for the original isolation of virus. However, my* experience has been favorable. In Colombia, in approximately 40 isolation experiments in both rhesus monkeys and mice, when in many cases the virus was from mosquitoes rather than from man, there were only two instances in which evidence of infection was observed in the monkey without manifestation in the corresponding mice. More recently, in Nigeria, West Africa, during the study of an outbreak of yellow fever, 34 isolations of virus from human material were made in mice alone with little difficulty In most instances, all mice inoculated became ill in a *regular manner.*

The experience in the laboratory at Entebbe, Uganda, has not been so satisfactory in this respect. In several instances, virus has been obtained by monkey inoculation when none has been noted in the mice inoculated with the same material.

Notwithstanding these occasional discrepancies, it appears that isolation in mice alone is a reasonably dependable procedure, especially in human suspect cases

When the rhesus monkey is used, there may be the rare instance in *which no circulating virus is demonstrated although the animal may* have been infected with or without a resultant temperature rise. Under such circumstances, blood serum taken a month or so after inoculation may be tested for specific antibodies in comparison with the pre-experimental serum. The presence of specific antibodies in the second specimen when these were absent from the first is indicative of a yellow fever infection of the animal in the interval between the examination of the two specimens. If all other possible sources of infection in the laboratory during this time can be eliminated, it may be concluded that virus was present in the original material

From what has been said, it is clear that the origin of a virus recovered can be ascribed to a particular inoculum only to the extent that all other sources of virus have been eliminated. There is an uncanny tendency for unsuspected technical errors to occur at critical moments in an experiment. *It cannot be overemphasized that the only means of ensuring the* validity of a virus isolation is to have in the laboratory no virus source other *than the inoculum under study.*

B Demonstration of Specific Lesions

1. *Human cases.* In fatal cases of suspected yellow fever, autopsy may establish the diagnosis without delay. Grossly, there is evidence of profound parenchymatous lesion of the liver and kidneys with a

marked tendency to hemorrhages by diapedesis, particularly in the mucosa of the gastrointestinal tract. Save for these findings and a variable amount of jaundice, there is nothing striking A positive diagnosis cannot be made from the gross findings, although a diffuse fatty parenchymatous change in the liver may justify a strong suspicion

While the renal lesion may be as important as that of the liver in causing death, it is the liver that is pathologically diagnostic. Microscopically there is a diffuse, nonpurulent necrosis most prominent in the midzone of the liver lobule and further characterized by discontinuity in the liver cell cords and a tendency for the necrosis to be coagulative, giving rise to the so-called "Councilman bodies." Invariably there is a collar of surviving liver cells about the central vein of the liver lobule. Necrosis may and does involve all three zones, and in the fulminating case the process may be so extensive that zonal predilection is lost.

Special fixations and stains are unnecessary The usual formol fixation with hematoxylin and eosin staining is entirely satisfactory. A well-prepared frozen section will enable the diagnosis to be made at once

C Demonstration of Increasing Antibody Titer in Convalescence

When recovery occurs the histologic method cannot of course be applied Furthermore, it happens frequently that the question of possible yellow fever does not arise until several days of illness have elapsed so that attempts to isolate virus are unsuccessful Diagnosis can still be made by utilization of the fact that the antibody content of the blood, which may be demonstrable with difficulty on the 5th or 6th day of the disease, rises rapidly during convalescence. On comparing the antibody content of acute and convalescent phase sera in yellow fever, there is found a great increase in antibody titer in the second specimen over that in the first

Serial dilutions of each serum are subjected to a standard protection test The technic may be either an intraperitoneal procedure such as that of Sawyer and Lloyd or the intracerebral method of Theiler. The second has the advantage of requiring a very small amount of serum.

When the first specimen is obtained on the first day or two of the clinical disease, the undiluted serum will have no neutralizing effect against yellow fever virus, and the second specimen may give an end point (50 per cent survival ratio) in a dilution of as high as 1:1,000. The absolute magnitude of the titers will depend on many factors in the conduct of the test, such as the amount of virus used and the contact

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II. SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE

A Intraperitoneal Protection Test

The intraperitoneal protection test involves the inoculation of a mixture of neurotropic yellow fever virus and the serum to be tested into the peritoneal cavities of a group of mice When adult mice are used, starch solution is also inoculated intracerebrally to produce a localizing lesion. All methods in current use employ the French neurotropic strain of virus fixed for mice. The technics of Sawyer and Lloyd,[1] Smithburn,[2] and Whitman[3] are presented below

1. *Adult mouse test* (*Sawyer and Lloyd*[1]).

a Specimens to be examined At least 6 ml of the serum to be tested are needed The test requires 3 ml The remainder is used in case the results of the first test are doubtful or the control tests prove unsatisfactory.

The mice Healthy young adult white mice of about 20 gm. weight are preferred, but moderate differences in age do not seem to affect the results of the tests

b Preparation of the virus An approximate time is set in advance for the tests, preferably 2 or 3 consecutive days in the middle of the week Five days before each testing date, a sufficient number of healthy mice are inoculated intracerebrally with yellow fever virus in mouse brain tissue. The mice are inspected each morning The definitely sick animals are killed with chloroform and are pinned out. The skin is slit with scissors from the nose to the middle of the back, and the flaps are laid back An assistant sears the top of the skull with a red-hot soldering iron The operator removes the bony covering over the brain with sterile, sharp-pointed scissors and then spoons out the brain with a rigid, narrow spatula of nickel The brain is placed in a small, weighed petri dish The other brains secured are placed in the same dish, and the total weight is ascertained The brains are then finely ground up in a porcelain mortar with enough isotonic salt solution, or preferably salt solution containing 10 per cent of normal serum, to make a 10 per cent suspension

c Preparation of the serum-virus mixtures In advance of the tests, 3 ml of each specimen of serum or diluted serum and of each of several sera for the controls are placed in a small test tube If less than 3 ml. of serum are available for a test, a 50 per cent solution of the serum may be

time, hence, the importance of including both the acute and convalescent phase sera in the same protection test.

If the first specimen has been obtained later in the course of the disease, it will have antibody, but the end point will be low; thus only a comparison of the end points will show whether there has been a rise in antibody content during the interval.

If the suspect disease is not yellow fever and the patient has not been previously immunized against yellow fever, neither the acute phase nor the convalescent phase serum will give protection against yellow fever virus, but if the patient has been previously immunized either by vaccination or by having had yellow fever, both sera will be protective and will give the same titer

D. Application of These Methods to Animal Studies

The three methods of diagnosing yellow fever which have been discussed above in their human application may be applied to studies in animals The most satisfactory is the immediate demonstration of virus by the inoculation of the infected animal's blood. It should be remembered that the sera of many species of animals are toxic to mice when inoculated intracerebrally, but the effect disappears on dilution, and a preliminary test will suffice to show the degree of dilution necessary. Dilution may be made with physiologic saline. Attention to this factor may be necessary in protection tests

Histopathology tends to be less useful in animal studies than in human cases Many monkeys exhibit lesions similar to those occurring in human patients, but these animals are largely of the species in which the disease tends to cause high fatality. Most of the species important in the maintenance of jungle yellow fever have the disease in a mild form and show no characteristic lesions in the liver. In fatal cases, lesions may be present, but the histologic criteria of the human disease cannot be applied It is essential to study the manifestations of the disease in each species by itself in order to develop diagnostic criteria.

The same comment applies to the interpretation of protection test results Many animal and bird species possess nonspecific neutralizing capacity for yellow fever virus upon which specific antibody behavior may be superimposed It is of even greater importance, therefore, that pre- and postexperimental sera be tested in the same protection test run and that the criteria of interpretation be based on the behavior of the particular species toward virus under controlled conditions

tive results are then obtained they are accepted, but the protection ratios for both tests are shown in the tabular reports to the senders of the sera The "unsatisfactory" results include those in which the controls are unsatisfactory and those in which fewer than 4 mice are alive and well 4 days after inoculation

GUIDE FOR INTERPRETATION OF THE INTRAPERITONEAL PROTECTION TEST IN MICE

No of mice living and well 4 days after inoculation	No of deaths or survivals allowed among these mice 5 to 10 days after inoculation if the report is to be "protection" or "no protection" respectively *	
	Protection (+)	No protection or negative (—)
	No of deaths allowed	No of survivals allowed
1, 2, 3	Result "unsatisfactory" (U) in any case	
4	0	0
5, 6	1	1
7, 8, 9	2	2
10, 11, 12	3	3

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2. *Adult mouse test of Smithburn.*[2] At the Yellow Fever Research Institute, Entebbe, Uganda, the routine test is a somewhat modified form of the method of Sawyer and Lloyd described above In the preparation of the virus, the infected mouse brains are ground in 10 per cent normal human serum in isotonic saline instead of saline alone as has become customary in many laboratories using the intraperitoneal test. This permits a 1 per cent virus suspension to be employed and results in considerably greater sensitivity with no loss in specificity.

The intraperitoneal inoculation of the serum-virus mixture and the intracerebral injection of starch solution are given almost simultaneously so that there is a constant relation between the times of the two inoculations This is an important factor, and it will be noted above that Sawyer and Lloyd recommended this procedure originally Nevertheless, it has been the general practice to inoculate the starch solution from 1 to 2 hours before doing the test, introducing thereby an additional source of variation and reducing the sensitivity of the test

tested if the dilution is stated in the report. To each tube is added 1.5 ml of the virus suspension. The contents of each tube are mixed and drawn up into a 5 ml. graduated glass syringe bearing either the number of the specimen or that of the mouse group to be inoculated.

d. The starch solution. The starch solution for intracerebral injection is prepared in advance by adding 2 per cent of cornstarch to isotonic saline and heating in a water bath. The solution is then placed in small, wide-mouthed Erlenmeyer flasks, autoclaved, and stored ready for use.

e. Controls. With each set of tests there should be 5 control groups of 6 mice each. Two groups should receive 0.4 ml. of normal serum (human or monkey) in place of the unknown serum of the test, and two should receive a known immune serum (human or monkey). To conserve the immune serum, it may be diluted to 10 per cent if of sufficiently high titer. The 5th control should be given the virus mixture intracerebrally. It shows whether the mice are susceptible and the virus potent. Intracerebral inoculation brings the animals down 1 day sooner than intraperitoneal inoculation with simultaneous cerebral injury.

f. Inoculation of the mice. An assistant anesthetizes the mice. The operator injects 0 03 ml of the starch solution intracerebrally, using a tuberculin syringe and fine needle. Then, with one of the larger syringes, an intraperitoneal injection of 0.6 ml of the virus-serum mixture is given. The mouse is then dropped into a numbered jar. The actual injections require approximately 3 minutes for each mouse group.

g. Inspection and recording. The mice are inspected every morning, and a record is made of those that are sick or dead Printed cards, 3 inches by 5 inches, such as shown in Fig. 1, may be used. The first mouse to become sick or die becomes Mouse 1 of the group, and the second, No 2, and so forth.

h Interpretation of the results. The result of the protection test is recorded as the ratio of the number of mice surviving on the 10th day after inoculation to those that were alive and well on the 4th day. Deaths before the 5th day are in all probability not due to the yellow fever virus. From the record cards, the results are classified as "protection," "no protection," "*inconclusive*," or "*unsatisfactory*," *in accordance* with the requirements of the guide (table, p. 382ff). Ordinarily, a specific death later than the 10th day shows a considerable protective action of the serum and has almost the same significance as a survival. If the amount of serum will permit, the tests of the specimens in the "inconclusive" and "unsatisfactory" groups are repeated, and if definitely positive or nega-

c. Controls Each protection test run has 2 immune serum controls from different donors, 2 nonimmune serum controls from different donors and 1 virus titration control The virus is titrated in 4-fold dilutions (1 1 through 1 256) using 12 mice for each dilution At times a standard immune serum pool is titrated as a control in 4-fold dilutions, using a range sufficient to cover the end point

d Inoculation of mice Groups of 6 white Swiss mice 19 to 20 days old and weighing 9 to 10 gms each are injected intraperitoneally with each serum virus mixture as soon as all the tubes have been prepared and in the order in which the virus was added to the test specimens A three-man team gives the best results—one to fill syringes, a second to anesthetize the mice, and the third to inoculate the mice and carefully check all the serum and mouse group numbers with the work list

e Interpretation of results With the assumption that the controls are satisfactory, interpretation of the results are given in the following table from Whitman's paper

INTERPRETATION OF RESULTS OF INTRAPERITONEAL PROTECTION TESTS BASED ON SAWYER AND LLOYD[1]

Number of Mice Living on 4th Day*	Number of Mice Living on 10th Day		
	Negative	Inconclusive	Positive
4	0	1, 2, 3	4
5	0, 1	2, 3	4, 5
6	0, 1, 2	3, 4	5, 6

* With less than 4 mice alive on the 4th day, the test is unsatisfactory

B. INTRACEREBRAL PROTECTION TEST

1. *Method of Theiler.*[5] The intraperitoneal methods previously described all employ unstandardized virus suspensions of fresh mouse brain in saline or serum saline The standard test also requires 3 ml of serum, an amount which makes it ill-adapted to studies in small animals. The intracerebral technic of Theiler employs a standardized desiccated virus preparation so that a predetermined dosage can be given and the sensitivity thus regulated within considerable limits With highly susceptible mice, a very sensitive test is obtained with a virus dose of the order of 25 LD_{50} (50 per cent mortality doses) whereas one of the order of 500 LD_{50} will give results comparable to those of the standard intraperitoneal test of Sawyer and Lloyd The serum required is 0.3 ml.

a Procedure. The procedure given here is that of Theiler modified in minor respects by Bugher[6] and employed at Bogotá, Colombia, and

All other features of the test are the same as described for the standard intraperitoneal procedure

3. *Young mouse test* (*Whitman*[3]). This procedure is being employed routinely by the United States Public Health Service in its laboratory at Hamilton, Montana The technic is essentially the same as described by Whitman in his original publication of the method.[3] In addition to its greater sensitivity, the test has the further advantage of employing less serum than the procedure of Sawyer and Lloyd. Amounts as small as 1 ml. of serum may be tested satisfactorily.

The procedure being followed at Hamilton[4] is as follows:

a. Test virus. The French strain of neurotropic virus (400th to 500th passage in mice) is used. A suitable number of adult white Swiss mice approximately 50–60 days old are each injected intracerebrally with 0.03 ml of a 6 per cent suspension in physiologic saline of freshly prepared mouse-brain virus Three days later all the living mice are killed with chloroform, and the brains are removed with as little contamination as possible. Considering each brain to weigh 0 4 gm , a 20 per cent mouse-brain suspension in 10 per cent nonimmune serum-saline is prepared This is done by using a sterilized enamel bowl (approximately 8 inches by 4 inches deep) and sash brush with bristles about 2 inches long. Working the brush up and down and to the sides for several minutes will grind the brains much finer than a mortar and pestle. The diluent is next thoroughly mixed in, adding it slowly at first; then the virus suspension is centrifuged at 3,500 r p m. for 30 minutes The supernate is transferred to a flask Cultures are made after centrifugation.

b Serum-virus mixture The optimal virus dosage for each mouse is approximately 8 LD_{50} This dose is usually contained in 0.02 to 0 04 ml. of the 20 per cent virus suspension described above.

The volume of serum for each mouse to be mixed with the virus is routinely 0 11 ml , although this may be varied at will from 0.04 ml. to 0.36 ml , depending on the amount of serum available and the sensitivity of the test desired The serum virus mixture is made up on a basis of 10 times volume received per mouse in order to allow for injection of 6 mice, possible injection of a 7th, and loss in manipulation

In advance of preparing the virus, the sera to be tested are measured into tubes bearing the respective serum serial numbers Then the virus is carefully added to the side of each tube at a point about ½ inch above the serum Each tube is agitated so that there is complete mixing of the serum and the virus, and no isolated drops of either serum or virus are left on the side of the tube.

Virus is added to all of the tubes, including those containing the control sera, in exactly the same amount and manner A portion of the virus suspension is put aside in another tube for titration at the end of the test. This portion, about 2 ml, is allowed to stand with the test.

The entire rack of tubes is thoroughly shaken for about 30 seconds as an added means of ensuring complete mixing, and it is then allowed to remain for the desired contact time without further attention The contact time and temperature used are 2 hours at 37° C.

With the nominal contact time of 2 hours, injection of the first mixture is begun 1¾ hours after adding the virus Ten or 12 tubes are taken from the rack, and the remainder are placed in the refrigerator. Under uniform ether anesthesia the injections are made with a ½ ml tuberculin syringe equipped with a No 27 gauge, ½-inch needle As many sterile syringes should be ready as there are serum mixtures to inject One finds a relatively soft area in the skull of the mouse in the parietal region, through which the needle may be pushed very easily. A penetration of 2 or 3 mm into the brain substance is sufficient. Deeper injection is likely to kill the mouse from pressure about the midbrain and brain stem, which is also true of inoculations carried out more posteriorly Inoculation into either the right or left cerebral hemispheres will give a minimum of traumatic deaths Ordinarily, 6 mice are inoculated with each mixture, but where finer discrimination is desired, 12 mice may be used.

As soon as the first lot of mixtures is injected, a second is removed from the refrigerator, and so on until all have been inoculated The purpose of this is to give as nearly the same time conditions to all mixtures as possible, the first tubes receiving somewhat less than the 2 hours and the last ones somewhat more By placing the remaining tubes in the refrigerator, a further attempt is made to minimize the effect of the time lag Since the addition of the virus takes about 15 minutes and the injection requires 1 hour, the last tubes will have had slightly less than 2½ hours of contact time, 45 minutes of which will have been spent at low temperature Ideally, the test should be injected in the time required to add the virus, but practically, this is not necessary

Smoothness in performance depends upon well-trained assistants. In addition to the operator, an anesthetist and one other assistant are needed. Greater accuracy and speed are obtained if the mice have been previously placed in properly numbered boxes or jars

The details of the test are entered on the standard mouse cards and also in a separate protection test book. The cards are used for the actual

at the former Yellow Fever Research Institute, Lagos, Nigeria. The test is also employed in a similar form but differing in minor features in the laboratories of the Yellow Fever Service, Rio de Janeiro, and in those of The Rockefeller Foundation, New York City The differences are chiefly in the matter of contact time and temperature and the strain of mice used. The procedure is as follows.

Into a sterile Wassermann tube, 0.3 ml of the serum to be tested is pipetted. The sera are grouped according to considerations detailed in a following section, and a sufficient number are selected to compose a "run." The standard run is of 60 sera, exclusive of controls. It is preferable to limit runs to a size permitting injection in not more than 1 hour.

The first control is made up of 5 tubes of normal human or monkey serum, or normal serum of the particular species of animal if but one species is being studied over a period of time. The second control is a known immune serum which is preferably entered in the test as a titration; that is, serial dilutions are prepared using the standard diluent and 0 3 ml amounts of each of the dilutions entered in the test. Dilution ratios of 4 or 5 should be used and two dilutions above and two below the anticipated end point entered in the test. This necessitates, of course, preliminary standardization of the immune serum.

When all sera have been prepared for the addition of virus, they are listed in order on a work sheet, and opposite each serum are entered the numbers of the mouse groups which are to receive the respective serum mixtures. This sheet is the guide for the remainder of the procedure and greatly facilitates speed and accuracy in the final injection of the material.

All being in readiness, 2 tubes of desiccated French neurotropic virus of an accurately standardized lot are rehydrated, and a dilution is prepared which should have the calculated quantity of virus desired for the particular test, bearing in mind that, in making the mixtures, the virus is diluted 1:2. To each tube 0.3 ml. of this virus dilution is added, a 1 ml. pipette being employed and the tip of the pipette being allowed to touch the interior surface of the tube about 1 cm above the contained serum. On removing the pipette the tube is tipped and agitated so that the serum washes every place touched by the virus, and the two are thoroughly mixed. This step is very important as the persistence of a droplet

tube individually, but it can be done very rapidly.

pipette to ensure accuracy in volumetric measurement. The virus is then frozen and dried *in vacuo* by the technic described by Sawyer, Lloyd, and Kitchen[8] and by Lloyd and Penna[9] or the more recent method of Bauer and Pickels[10]

(3) Diluent The standard diluent used is 10 per cent normal rhesus monkey serum in 0 9 per cent saline solution The monkey serum is a pool made from sera of monkeys known to be negative in a sensitive protection test After making the pool, it is again tested as diluent by allowing the virus dilutions made in it to stand 2 hours at room temperature, titrations in mice being made at the beginning and end of the period The loss of titratable virus should not exceed 20 per cent in this period

As a precautionary measure, the pooled normal serum is filtered through a Seitz pad and dispensed in 5 0 ml ampules for use Diluent is then made up as needed with sterile saline, no further filtration being necessary

Alternatively, 0 5 per cent bovalbumin in 0 9 per cent saline may be used as the virus diluent It has given satisfactory results with substantial economy in comparison with serum

(4) Volume of serum used The amount of serum used in this test is the least amount that permits the injection of 12 mice If less than 0 3 ml of serum is available, it may be augmented in volume by the addition of standard diluent to equal 0 3 ml, and a note should be made that the test was not carried out on the whole serum

(5) Contact time The importance of the *in vitro* contact time has not been sufficiently emphasized in discussions of protection tests In the intracerebral test, satisfactory and fixed contact time is fully as necessary as standardized virus

(6) Controls The controls used in this test are three

Virus titration A sample of the virus dilution used is allowed to stand with the test, and at the end a titration is made Since the value of the virus is already approximately known, it is not necessary to cover a wide range in this titration

This virus titration thus represents the original virus plus whatever effect on it there may have been because of the diluent With a well-stabilized virus preparation, an aberrant result in the titration, if no technical error has occurred, should lead to a re-examination of the diluent for virucidal activity

Immune serum A pool of known immune serum, either human or monkey, is desirable for this purpose Where extensive work is being

mouse record. The duplication of these entries in the special book is worth the extra effort later when analysis of the results is carried out.

The mice are observed each day at the same hour, and the sick and dead are recorded On the standard card, the first mouse to die becomes No 1 and so on. The observations are continued for 10 days, when all mice are discarded after the 10th day reading

b. Materials and controls. In the foregoing outline, it is evident that at every step are factors which can and do lead to variability in the results. It therefore becomes highly important to standardize each factor in order to minimize the over-all variability The extent to which this is achieved determines the applicability of the test to the demonstration of small amounts of antibody

(1) Mice As in the intraperitoneal test, it is highly important that the susceptibility of the mice be maximal and as uniform as possible from mouse to mouse If, after a titration of French neurotropic virus in the mice, a dilution is prepared which contains 10 LD_{50} as calculated by the method of Reed and Muench[7] and a new lot of mice is inoculated intracerebrally with 0 03 ml , they should, with very rare exceptions, all die. Almost all of the deaths will occur on the 5th to 7th day.

If the strain of mice used in any laboratory fails to give such uniform results on testing with virus, protection tests should not be started until a good strain is available It is also wise to compare the susceptibility of a strain of apparently good mice with that of well-established colonies in other laboratories through the exchange of desiccated virus shipped in thermos bottles and packed in ice and carbon dioxide.

We prefer to use mice from 42 to 50 days of age in order to keep this factor uniform, although there are no clear data to the effect that older mice are not equally satisfactory.

(2) Virus. The virus is prepared as follows: Sufficient mouse brains infected with French neurotropic virus are removed to make 2.0 gms This material is then ground in a sterile mortar, and small amounts of normal rhesus monkey serum or normal human serum are added until a total of 40 ml. has been reached. The emulsion is divided in 50 ml centrifuge tubes and centrifuged at 2,500 r p m for 30 minutes, a shorter time at higher speed is better if the centrifuge permits The supernates are collected and pooled. No gross particles should be visible if the supernatant portions are carefully taken off. Filtration is not necessary. The virus is placed in 0 5 ml amounts in sterilized soft glass tubes 120 by 13 mm , or 5 ml. pyrex ampules The dispensing is done with a 1.0 ml

Two mice are inoculated intracerebrally with each dilution, using one syringe for the three mixtures beginning with the most dilute (front tube) The pairs of mice are spotted with yellow, blue, and red dyes in that order to distinguish the dilutions since all 6 mice are placed in one jar

After completing the inoculations of the test sera, the virus controls are completed by adding 0 5 ml of diluent to 0 5 ml of each of the two final virus dilutions Without further incubation, these mixtures are inoculated into 3 mice each Those from the 1 500,000 mixture are spotted red and those from the 1 1,000,000 are colored blue The 6 mice go into one jar All inoculations are 0 03 ml in amount and are made into the cerebrum without anesthesia

b Interpretation Since there are 2 mice for each dilution, it is evident that several patterns of result may occur in accordance with the following schedule

Virus Dilution	Mortality				
1 500	+	+	+	—	
	+	+	+	—	+ = Death
1 5,000	+	+	—	—	— = Survival
	+	+	—	—	
1 50,000	+	—	—	—	
	+	—	—	—	
Interpretation	0	+	++	+++	

Since the virus used kills regularly all the mice inoculated with the mixture made from the 1 500,000 virus (the final dilution is 1 1,000,000 after the diluent has been added) and is frequently irregular in the succeeding one which is twice this dilution, the dilution which kills all of the mice is said to have 1 m l d so that in the test the three dilutions may give dosages of 600, 6,000, and 60,000 m l d per mouse.

The interpretation symbols have the following meaning therefor

- 0 No protection
- \+ Feeble protection Serum neutralizes 600 m l d approximately
- ++ Strong protection Serum neutralizes 6,000 m l d approximately
- +++ Very strong protection Serum neutralizes 60,000 m l d approximately

As in the tests described previously, the mice are observed for 10 days and the deaths recorded.

Special attention has been paid at Dakar to the testing of icteric sera, which are generally recognized as tending to give false neutralizations

done with one animal species, it is convenient to establish an immune pool of that species In any case, the pool should be large enough to permit its use over a prolonged period. Greater value is secured from this control if it is set up as an antibody titration, serial dilutions of the immune serum being carried out to both sides of the end point.

Nonimmune serum. Either as a separate control or included with the run itself, it is important to include a known nonimmune serum. Routinely, 5 tubes of known nonimmune serum are inserted in each run.

c. Interpretation The results may be classified according to the schedule given under the adult mouse test of Sawyer and Lloyd. This is the most commonly used procedure A somewhat finer measurement of the result may be obtained by the use of the "average survival time"[6] wherein the time of death is considered in addition to the fact of death or survival. For diagnostic purposes, the use of the survival ratio is entirely sufficient. Those who may desire to use the average survival time criterion are referred to the published discussion of the method.

2. *Method of the Pasteur Institute, Dakar, French West Africa.*[11] This technic differs in several respects from any other in common use today and, because it has been used in the large-scale vaccination program in French West Africa and in comparative studies of vaccine efficiency, it merits inclusion in this manual

a Procedure The virus source is the desiccated whole brain substance of mice which have been infected with neurotropic yellow fever virus. Two such brains are ground in a mortar with 4 ml of 10 per cent normal serum saline (human or monkey). This suspension is called a dilution of 1 5. From this, further dilutions in 10 per cent serum saline are made. 1 500, 1 5,000, 1.50,000 for the test itself and two additional dilutions 1·500,000 and 1:1,000,000 for the virus control

Three tubes are used for each serum, and these are placed in a rack holding 25 columns of 3 tubes All tubes of the front row receive 0 5 ml of the 1:50,000 virus, the middle row receives 0 5 ml. of the 1:5,000 virus; the 1:500 dilution is dispensed in the same amount to the tubes of the back row.

The sera are then added. Beginning with the front tube (containing the most dilute virus) 0 5 ml of the serum to be tested is added to each tube from front to back. As the mixtures are made, each tube is shaken individually to ensure that all virus is in contact with serum The rack is then allowed to stand ½ hour at room temperature (25°–30° C.).

without antibody content should die, while all those injected with antibody-containing mixtures should survive. A brief experience with animal as well as with human sera following known infection with yellow fever or vaccination with living virus is sufficient to demonstrate that the virus-neutralizing capacities of such sera are quite variable Muench,[12] from statistical considerations applied to a large mass of data, has shown that the mortality distributions encountered are compatible with the expected frequencies in a complex sampling field made up of negative sera with a single mortality probability and positive sera whose corresponding mouse mortality probabilities themselves form a distribution, although the exact form of the distribution may not be known

In the presence of such a situation, combined with variations inherent in the test itself, it is quite evident that the decisive differentiation between negative and weakly positive sera contains definite elements of arbitrariness. As in any quantitative procedure, as the variability rising from technical sources is reduced, more precise judgment may be exercised with respect to the phenomenon being measured Also the working standard should be so established as to keep the significant errors at a minimum

This last consideration has usually not been stressed sufficiently in regard to yellow fever protection test results For example, when human surveys are made to determine the regions in which yellow fever exists or has existed, it is highly important that the sera reported as positive should be true positives and indicate, beyond doubt, that the donors have had the infection The matter of failing to demonstrate weak posi- [illegible]

strongly positive Therefore, for such purposes, a test with an abundance of virus may be desirable, with the sacrifice of maximum sensitivity.

If the object is to determine whether any antibodies whatever exist, as in postvaccination studies or in animal experimentation, the failure to detect a weak positive may be as great an error as the report of a false positive The standards in a case of this kind should be such as to make the totality of errors minimal

It is to be emphasized, especially with animal sera, that the standards of interpretation must be established by the study of sera from known infected and noninfected animals Essentially, this involves an extensive

With such sera, the protein is separated and then reconstituted, eliminating the biliary products that tend to give false reactions. False and true reactions can thus be distinguished.

C. Comment

The mouse protection tests described here are in use in various laboratories where yellow fever is being studied, and they form the basis for many publications which have appeared. It is important to realize that there are differences between them in such matters as sensitivity. The test being used at Dakar is by far the most severe, in that a large amount of virus must be neutralized in order that all of the mice inoculated with a mixture may survive. The method of Sawyer and Lloyd as usually performed also has a low order of sensitivity although when the original technic is followed, it is considerably more sensitive The use of 1 per cent virus in 10 per cent normal serum saline with simultaneous starch injection and intraperitoneal inoculation gives a sensitivity of the same order as that of the intracerebral test of Theiler and of the young mouse test of Whitman The 1 per cent test, however, requires 3 ml. of serum as compared with 0 3 ml needed for the intracerebral method. It will be found that the sensitivities of all these methods will differ in various laboratories, partly from variations in technic but especially from differences in mice.

With the exception of the method of Whitman, none of those given requires mice of critical age Very satisfactory tests may be made in suckling mice, but such methods have not been included since they presuppose the existence of a mouse colony and are applicable only in highly specialized circumstances.

Similarly, other serologic methods, such as complement fixation, have not been included These tests have not the conclusive diagnostic value of the neutralization reactions and have not as yet the general applicability

It should also be recognized that the schedules of interpretation which have been given really apply specifically to human sera. It will be found that they may be used with satisfaction for nearly all monkeys and apes as well, but their extension to orders other than the primates should be made with care.

At first thought, one is inclined to say that a properly established protection test is one in which all mice inoculated with serum mixtures

517 Mouse Group		26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
1137 Serum	1												†				
	2															ǂ	
F-142 Virus	3															ǂ	
	4															ǂ	
Control for	5															ǂ	
	6															ǂ	

Source of Virus See card 511

Result — Mouse Strain K — Form 381

Serum																	
	2																
	3												†				
F-142 Virus	4															ǂ	
Control for	5															ǂ	
511-528	6															ǂ	

Source of Virus See card 511

Control Group — Inoculum Intracerebral-Starch 2%, 0.03cc.

Intraperitoneal - Virus 20% 0.2 cc

(5/5) Result — Mouse Strain K. — Serum 100% 0.4 cc. — Form 381

Result — Mouse Strain K. — Serum — Form 381

Key to symbols used in records

† = Died

‡ = Killed when sick

ǂ = Killed when well

S = Sick

M = Moribund

F-142 = French strain of virus, 142nd passage in mice

6/6 (1/11) = 6 of 6 mice were protected in test; 1 of 11 survived in normal serum controls

\+ = Serum protected

± = Result inconclusive

− = Serum did not protect

U = Test unsatisfactory

Heavy vertical lines bound time zone within which deaths are considered significant

FIG 1 Part of record of test of a serum by the intraperitoneal protection test in mice to determine protective power against yellow fever Upper card contains record of test other two show that of half the immune and normal serum controls

study of the variability of results in animals known never to have been in contact with yellow fever virus but which may be presumed to reflect all other factors operating in the region under consideration.

A further word of caution concerns the general technical background for these as well as all other tests involving virus neutralization. All glassware must be meticulously cleansed if one is to avoid introducing systematic errors resulting in false neutralizations. Residues of alkaline washing powders and soaps as well as of oxidizing cleaning agents may destroy the virus and give false reactions, particularly with the methods employing small amounts of serum in which the wetted surface of the glassware is relatively large in comparison with the volume of the liquid. If great certainty is desired concerning any one result, the test should be repeated, preferably with another bleeding of the same donor.

III. REFERENCES

1 Sawyer, W A, and Lloyd, W The Use of Mice in Tests of Immunity Against Yellow Fever *J Exper Med*, 54 533–555, 1931.

2 Smithburn, K C Experimental Studies on the Yellow Fever Protection Test *J. Immunol*, 51 173–189 1945

3 Whitman, L Modified Intraperitoneal Protection Test for Yellow Fever Based on Greater Susceptibility of Immature White Mice to Extraneural Injection of Yellow Fever Virus *Am J Trop Med*, 23 17–36, 1943

4 Personal communication, Rocky Mountain Laboratory, U.S. Public Health Service

5 Theiler, M A Yellow Fever Protection Test in Mice by Intracerebral Injection *Ann Trop Med*, 27 57–77, 1933

6 [illegible] Animal Sera by the [illegible]–841, 1940

7 [illegible] Per Cent End [illegible]

8 [illegible] of Yellow Fever [illegible]

9 Lloyd, W, and Penna, H A Preservation of Yellow Fever Immune Sera *Am. J Trop Med*, 13 291–295, 1933

10 Bauer, J H, and Pickels, E G Apparatus for Freezing and Drying Virus in Large Quantities under Uniform Conditions *J Exper Med*, 71 83–88, 1940

11 Personal communication, Pasteur Institute, Dakar Dr. Peltier, Dr. Durieux, and Dr. Joncher

12 Muench, H The Probability Distribution of Protection Test Results *J Am Stat Assoc*, 31 677–689, 1936.

DENGUE

ALBERT B. SABIN, M.D.

Professor of Research Pediatrics, University of Cincinnati College of Medicine and The Children's Hospital Research Foundation, Cincinnati, Ohio

I INTRODUCTION

DENGUE is an infectious, mosquito-transmitted disease, characterized, as a rule, by fever of 5 to 7 days' duration, by pain in various parts of the body which may be severe enough to lead to prostration, and by morbilliform, scarlatiniform, or petechial rash, lymphadenopathy, and leukopenia In nature the only known mode of transmission is by mosquitoes of the genus *Aëdes* and the only species incriminated thus far are *aegypti, albopictus, scutellaris,* and *polynesiensis.* Dengue is suspected, therefore, whenever a disease having some or all of the clinical manifestations just mentioned occurs at a time when these mosquitoes are present in appreciable numbers. Until recently it was generally believed that very little immunity followed a single attack of the disease It is established, however, that at least 2 immunologic types of the virus exist and that immunity to homologous types can be of long duration. It is

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noteworthy, however, that infections caused by heterologous immunologic types, especially within a few months after the first attack, may produce illnesses that are mild, without rash, and associated with fever that may be only of 1 to 3 days' duration.

Dengue virus is present in the blood in large amounts during the first 24 hours after onset of fever but, by inoculation of susceptible human beings, can be detected in diminishing concentration as long as the fever persists The virus as it is contained in human serum is readily filtrable, is not larger than 17 to 25 mμ, as determined by filtration through gradocol membranes, and can be preserved for at least 8 years either in the frozen state with the aid of dry ice or in the dry state after proper lyophilization The human virus produces an inapparent infection in monkeys, and, as a rule, is without effect in the ordinary laboratory animals or embryonated eggs Several strains of both immunologic types of the virus have now been adapted to mice. The mouse-adapted dengue virus is infectious only by the intracerebral route and may be differentiated from other known viruses by its limited host range in laboratory animals. The mouse-adapted dengue virus is unique in being pathogenic for mice but not for mature cotton rats, hamsters, rabbits, or guinea pigs. After approximately 100 passages in mice it was possible to cultivate the virus in chick embryos in continuous serial passage After a large number of intracerebral passages in mice, various strains of dengue virus acquired the capacity to produce in intracerebrally inoculated monkeys a paralytic disease which clinically and histologically is similar to poliomyelitis. Quantitative studies have shown that the monkey paralytogenic property was newly acquired during the course of the many intracerebral passages in mice and is not present in potent preparations of both immunologic types of the virus at earlier passage levels in mice (Sabin and Sweet, see Sabin [14])

After a certain number of passages in mice, both immunologic types of the virus have retained the capacity to produce a rash but have lost the capacity to produce the other clinical manifestations of dengue in human beings. Laboratory workers should remember, however, that the highly potent human virus present in the serum of patients shortly after onset of fever can produce the disease after instillation in the nose or conjunctival sac or after being rubbed into the scarified skin. A laboratory infection has been reported after infected human serum was accidentally squirted into a worker's eyes.

Repeated passage of dengue virus in 3- to 7-day-old suckling mice

yields brain suspensions with titers of 10^{-6} to 10^{-8}. Complement-fixing (C-F) antigen of adequate potency can be prepared from the brains of suckling mice having a high virus titer but not from weaned mice, which, as a rule, contain about 100 times less virus Human beings, rhesus monkeys, and chimpanzees infected with homotypic, human virus develop C-F antibody in titers of 1:128 to 1 256 Experimentally infected human beings residing in dengue-free areas still had C-F antibodies in titers of 1:2 to 1 128, 3 to 4 years after inoculation. The C-F antigen is not type-specific. Human beings and monkeys inoculated with heterotypic strains of human dengue virus yield convalescent sera which contain high titers of C-F antibody but little or no neutralizing antibody for the heterotypic virus Positive C-F tests have been obtained with the sera of people who had clinically diagnosed dengue in Hawaii, Guam, Japan, Java, and New Guinea 1 or more years before bleeding.

The dengue viruses have been shown to be antigenically related to the viruses of West Nile fever, yellow fever, and Japanese B encephalitis by means of the complement fixation test This relationship is demonstrable when concentrated heterologous C-F antigens are used, but usually not with diluted antigens containing the 4 to 8 units ordinarily used for diagnostic purposes The relationship among these viruses is usually not evident in neutralization tests, although monkeys have been encountered which *developed* heterologous neutralization indexes of 300 to 500 in their undiluted serum after infection with certain strains of *human* dengue virus Although no antigenic relationship was found between the viruses of dengue and St Louis encephalitis by means of the C-F test, a relationship was demonstrated by means of the hemagglutination-inhibition test Patients recovering from experimental infection with West Nile fever virus yielded sera with the most marked crossing with dengue hemagglutinins, whereas the sera of patients recovering from St. Louis encephalitis reacted with dengue in lower titers These cross reactions do not preclude an accurate diagnosis by serologic methods, but they emphasize the need of using multiple antigens, particularly in regions where several of these viruses may occur.[1, 10]

II METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

Recovery of a strain of virus from either human serum or mosquitoes may be attempted by inoculating suckling mice (2 to 4 days old) intracerebrally. The mice should be of the so-called Swiss variety because at

least one variety of white mice (PRI) has been described which is genetically resistant to the dengue–yellow fever–West Nile–Japanese B group of viruses.[11] The serum should be tested in the 1:10 or 1:100 dilution as well as undiluted, because of possible interference by large concentrations of virus particles not virulent for mice. The mosquito suspension should be rendered free of pathogenic bacteria by means of penicillin and streptomycin (about 1,000 units of each per ml. may be necessary) and only lightly centrifuged to remove gross particles. 0.01 ml. is injected intracerebrally. It is advisable to use at least 2 litters of suckling mice for each dilution. Some strains of virus will paralyze a large number of mice within 9 to 21 days; others will affect only an occasional mouse or none at all. If any of the mice succumb, the spinal cord and brain should be passaged in suckling and adult mice—one group intracerebrally and another intraperitoneally. If all the adult mice succumb by any route or if the intraperitoneally inoculated suckling mice succumb, the newly recovered virus is not likely to be dengue. If only the intracerebrally inoculated suckling mice succumb, the brains and spinal cords should be harvested for serologic identification of the virus by either neutralization or hemagglutination-inhibition tests described in the next section. For hemagglutination the tissue is ground up in a special buffer; material that is to be stored in the frozen state for subsequent use in neutralization tests should be ground in heated (56° C. for 30 minutes) undiluted rabbit serum. Strains of dengue virus, which paralyze few if any mice, produce an inapparent immunizing infection. The presence or absence of infection with either Type 1 (Hawaiian strain prototype) or Type 2 (New Guinea "C" strain prototype) dengue viruses in surviving mice is determined by an intracerebral challenge with 100 LD_{50} of the mouse-adapted strains which are highly pathogenic for adult mice; this is administered 4 to 6 weeks after the original inoculation in the suckling mice

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS

Antibodies for the dengue viruses can be demonstrated by neutralization tests in mice, and by complement fixation and hemagglutination-inhibition (H-I) tests. Since these tests have not as yet been applied to the diagnosis of naturally occurring acute infections, the only current guide to diagnosis is based on a study of Sweet and Sabin[12] on 4 experimentally inoculated patients who were observed over a period of 4 months after inoculation with human Type 1 (Hawaiian strain) or Type 2 (New Guinea "C") dengue viruses. Homotypic neutralizing and H-I antibodies

appeared within 7 days after the first rise in temperature, and homotypic C-F antibody appeared 2 to 3 weeks after onset All 3 varieties of homotypic antibody persisted for at least 4 months, and it is known from studies on other experimentally inoculated patients residing in dengue-free regions that the neutralizing and C-F antibodies can persist for many years without exposure to new infection Heterotypic neutralizing antibody, generally in much lower titer, appeared as early as the homotypic in 3 of 4 patients, was delayed for 1 week in the 4th patient, and persisted in all for at least 4 months Heterotypic C-F antibody, in a titer of 1 4 or more, appeared in all but 1 of the patients. The homotypic and heterotypic C-F titers were so similar in the patients infected with the Hawaiian strain that a diagnosis of the immunologic type of virus responsible for the infection would have been impossible or misleading by means of the C-F test In the 2 patients infected with the New Guinea "C" strain the C-F test permitted a type-specific diagnosis. All sera were also tested in dilutions of 1.4 and higher with 128 units of Japanese B encephalitis C-F antigen, and only 1 of the 4 patients developed a transitory rise to a titer of 1.4 to 1 8, which was very much lower than the level of homotypic dengue C-F antibody The heterotypic H-I antibodies appeared 1 week later than the homotypic and in sufficiently lower titer in 3 of the 4 patients to permit a type-specific diagnosis In the 4th patient the heterotypic H-I antibody appeared as early as th [illegible]

antibody provides the diagnostic test of choice, and that by using the 2 immunologically distinct dengue antigens, serologic confirmation of the clinical diagnosis is possible within 7 days after onset of fever.

A. Hemagglutination-Inhibition Test

1. *Time of collection, storage, and preparation of serum for test* The first specimen of serum should be collected as soon as possible after onset of fever, and the second specimen 7 days after onset of fever. The serum obtained and separated under aseptic conditions can be stored in an ordinary refrigerator and shipped without refrigeration. The specific antibody is separated from the normal inhibitor present in all sera by precipitation with acetone in the following manner:

(1) To 0 2 ml of serum in a graduated centrifuge tube add 0 8 ml of 0 9 per cent solution of NaCl, mix, and heat at 56° C. for 30 minutes

(2) Add 12 ml of acetone at room temperature, stopper with sterile cork, shake,

centrifuge for 1 minute at about 2,000 r p m Decant supernatant liquid, add 12 ml of fresh acetone, resuspend precipitate with wooden applicator stick, shake tube, and centrifuge at 2,000 r p m for 5 minutes Decant supernatant liquid, spread precipitate over lower portion of tube by means of an applicator stick, and leave tubes open at 37° C or room temperature until residual acetone has evaporated and precipitates appear dry

(3) Add 1.8 ml of 0 02 M phosphate buffer, pH 7 7, and dissolve precipitate with aid of applicator stick Add additional buffer to final volume of 2 ml and after precipitate appears completely dissolved, centrifuge at 2,000 r p m for 3 to 5 minutes to remove any insoluble residue The supernatant liquid is used in the test and is regarded as the 1 10 dilution of serum

2 *Preparation, titration, and storage of hemagglutinins.* Hemagglutinins for chick erythrocytes can be recovered from the brains of suckling mice infected with either Type 1 or Type 2 dengue viruses by extraction with M/64 borate-KC1 buffer, pH 9, in saline.[9,15] The optimum conditions for reaction with the erythrocytes and stability at certain pH and *temperature ranges vary with the type of virus and the number of passages* it has had in mice. Although hemagglutinins of high potency (titers of 1.2,560 or higher), reacting at both 23° C. and 4° C., were derived from both the 7th and 118th mouse passages of the Hawaiian strain, the pH zone for reaction was broader for the 118th passage material, particularly at 4° C Freshly prepared hemagglutinin derived from the 19th mouse passage of the New Guinea "C" strain gave no reaction at pH 6 5–6.9 at 23° C., which is optimum for the hemagglutinin derived from either the 7th or the 118th mouse passage of the Hawaiian strain, but yielded titers of at least 1:640 at pH 6.7 at 4° C Dengue antisera devoid of neutralizing antibody for the heterotypic virus contained high titers of *heterotypic hemagglutination-inhibition antibody.* Preliminary incubation of the hemagglutinin-antibody mixtures at pH 7.7 for 2 hours at 23° C to 25° C was necessary for the demonstration of maximum titers of antibody.

a. Preparation of hemagglutinins

(1) Viruses used Type 1. Hawaiian strain, mouse passage 117. Type 2. New Guinea "C," mouse passage 18

(2) Mice used. 3- to 5-day-old Swiss mice left with mothers.

(3) Inoculum 0 01 ml of 10 per cent centrifuged suspension of virus intracerebrally.

(4) Procedure When the majority of mice show CNS signs (5 days after inoculation for both viruses), all are killed by chloroform, and the

brains are harvested in the following manner (*a*) dip mice in 5 per cent phenol or other antiseptic solution, bleed out by cutting heart through chest wall and draining on absorbent cotton; (*b*) decapitate and squeeze brain into petri dish with the aid of a flat tissue forceps The weighed brain tissue is ground to a 20 per cent suspension with an abrasive (alundum) in a mortar (a motor-driven blender is not used because some hemagglutinins are destroyed by the agitation).

The fluid used for suspension is M/64 KCl-borate, pH 9, in saline (this buffer is prepared from 2 stock solutions (1) stock solution of 12 404 gm of boric acid, C.P., and 14 912 gm of KCl in 1,000 ml of distilled water, and (2) 0 2 M NaOH in distilled water To 563 ml of 0 9 per cent solution of NaCl add 50 ml of borate stock solution and 27 ml of 0.2 M NaOH) Centrifuge in cold room or in refrigerated centrifuge at an average G of about 7,000 to 10,000 for 1 hour—10,000 r p.m in Spinco LH, rotor 30, or 13,000 r p m in Sorvall Model SS-1 The supernatant liquid is removed, merthiolate is added to a final concentration of 1 10,000, and the preparation is stored at 4° C

The average yield from 100 suckling mice is about 75 ml In an alternate method of preparation described by Casals and Brown,[13] the brains are first submitted to a series of extractions in ethyl ether and acetone to remove normal inhibitor, and the suspension is then made in ordinary saline and centrifuged as described above

b. Titration of hemagglutinin. The reagents needed are chick erythrocytes and 0 02 M phosphate buffers in saline ranging in pH from 5 4 to 7 8 One- to 2-day-old White Rock chicks (other breeds should be tested before use with hemagglutinin of known titer) are chloroformed, decapitated, and the blood is allowed to fall into Alsever's solution (dextrose—2 05 gm, sodium citrate—0 8 gm, citric acid—0 055 gm, and NaCl—0 42 gm per 100 ml of distilled water, autoclave at 10 lbs for 10 minutes) About 1 ml of blood is obtained from each chick, it is collected in a flask containing 1 ml of Alsever's solution for each chick that one expects to bleed, e g, 25 ml of solution for 25 chicks After all chicks have been bled, the mixture is filtered through several layers of gauze, and enough Alsever's solution is added to provide 4 volumes of solution for each volume of blood This is stored at 4° C and is good for 1 week. The cells are washed in saline 3 times prior to preparation of a 10 per cent suspension in saline, which can be kept in the refrigerator for 2 days The 0 25 per cent cell suspension used in the test is prepared directly before use.

Since the alkaline extract representing the 20 per cent suspension (or 1.5 dilution of brain tissue) which is stored in the refrigerator, has

a pH about 8.0, it is necessary to use the following scheme to obtain the desired pH in the 1:20 dilution:

pH desired in first tube or 1:20 dilution	Add 0.25 ml of 20 per cent extract in borate buffer to 0.75 ml of 0.02 M phosphate buffer of indicated pH	pH desired in first tube or 1:20 dilution	Add 0.25 ml. of 20 per cent extract in borate buffer to 0.75 ml. of 0.02 M phosphate buffer of indicated pH
6.1	5.4	7.0	6.8
6.2	5.7	7.1	6.9
6.3	5.9	7.2	7.0
6.4	6.1	7.3	7.1
6.5	6.3	7.4	7.2
6.6	6.4	7.5	7.3
6.7	6.5	7.6	7.4
6.8	6.6	7.7	7.6
6.9	6.7	7.8	7.7

Chilled buffers of the desired pH are placed in 0.5 ml amounts in Wassermann tubes or plastic plates (the depressions must be absolutely smooth) which have been chilled for at least 20 minutes. 0.25 ml of the 20 per cent hemagglutinin is added to 0.75 ml. of buffer in the first tube or depression in the plastic plate according to the scheme indicated above and 2-fold dilutions are made by transferring 0.5 ml. amounts by means of an automatic pipette from tube to tube or depression to depression. One tube or depression containing 1 ml. of the buffer does not receive hemagglutinin and serves as a control. 0.5 ml of the 0.25 per cent suspension of chick erythrocytes in saline is then added to each tube or depression, and the racks or plates are placed at 4° C. for 90 minutes. A preliminary observation is made at 45 minutes to check on "slipping" patterns, but the last tube showing an approximately 50 per cent or 1+ agglutination pattern at 90 minutes is taken as the end point for calculation of the number of units to be used in the hemagglutination-inhibition test. When a 1+ reaction is not observed, the approximate 50 per cent end point is regarded as being midway between the last dilution showing a zero or ± reaction and the one showing a complete shield of hemagglutination.

The Hawaiian strain (Type 1) yields a hemagglutinin with a titer of about 10,240 at 4° C at the optimum pH of 6.7, and reactions of varying titer are seen in the pH range of 6.2 to 7.5. At room temperature the optimum pH is also 6.7, but the titer is 4-fold less. The 20 per cent extract

in borate-KCl buffer is stable for 4 months at 4° C. It can be preserved in sealed ampules in a box containing solid CO_2 but should not be refrozen after the first thawing because there is progressive loss of activity on repeated freezing and thawing The hemagglutinin is very unstable at pH 6.7, which is optimum for its reaction with the chick erythrocytes

The New Guinea "C" strain (Type 2) yields a hemagglutinin which shortly after preparation is demonstrable only at 4° C. in the narrow pH range of 6 5–6 7, with maximum titers at pH 6 6 and 6 7 The pH range widens with increasing storage in the refrigerator and the titer increases 4-fold or more, hemagglutination also occurs at room temperature This hemagglutinin should, therefore, be allowed to "age" at 4° C until it reaches maximum titers before it is used for hemagglutination-inhibition tests or is frozen Stability on storage is about the same as for the Hawaiian strain

3. *Procedure for hemagglutination-inhibition test* A duplicate titration of the hemagglutinin at pH 6.7 and 4° C. is performed on the day the test is set up, and the dilution required to yield 16 units per 0 25 ml. is calculated. This dilution is prepared in 0 02 M phosphate saline buffer, pH 7 7, after all serum dilutions have been prepared, because at this pH both hemagglutinins are stable at room temperature for at least 2 hours. To prepare the serum dilutions, 0 25 ml. of the pH 7.7 phosphate buffer is distributed in a series of tubes or plastic plate depressions The first tube of the series receives no buffer since it is reserved for 0.25 ml. of the dissolved acetone precipitate representing the 1:10 dilution of serum, 0.25 ml of this solution is also added to the second tube of the series to yield a 1.20 dilution, and further serial, 2-fold dilutions are prepared by transferring 0 25 ml amounts, preferably with an automatic pipette. The last tube of the series contains 0 25 ml of buffer and 0 25 ml of the dissolved acetone precipitate but no hemagglutinin. it serves as a serum control tube All the other tubes receive 0 25 ml. of the diluted hemagglutinin containing 16 units, and the mixtures are incubated at room temperature for 2 hours before addition of the chick erythrocytes The 0 25 per cent suspension of chick cells to be added to these tubes is prepared in 0.02 M phosphate saline buffer, pH 5 6. When 0.5 ml of this suspension is added to the mixtures at pH 7.7, the final pH is 6.7, which is optimum for hemagglutination. The tubes or plastic plates are then transferred to a refrigerator at 4° C. The patterns are checked at 30 to 45 minutes for evidence of "slipping" in tubes containing the lower dilutions of serum, and the final reading is made at 90 minutes. The last tube

showing no hemagglutination or less than a 50 per cent pattern is taken as the end point of inhibition The diluted hemagglutinin at pH 7.7, which remained at room temperature during the 2-hour period of incubation, is again titrated in duplicate to make certain that 16 units of hemagglutinin were still present at the end of this period.

Peak homotypic hemagglutination-inhibition titers *vs.* 16 units of hemagglutinin are usually 1:320 or higher. If titers of this magnitude are not achieved within 7 days after onset of fever, it is advisable to repeat the tests on a serum specimen obtained 2 or 3 weeks after onset. If the inhibition titers for both types of hemagglutinin are about equal and remain at 1 80 or less, it may be necessary to consider the possibility that infection was caused either by a hitherto unrecognized immunologic type of dengue virus or by some other virus that is antigenically related to dengue. The hemagglutination-inhibition test has been used successfully for serologic survey purposes on sera from Malaya and Borneo, and the different titers obtained with the Type 1 and Type 2 hemagglutinins permitted the conclusion that the Type 1 virus was prevalent in those regions.[14]

B Complement Fixation Test

1. *Time of collection, storage, and preparation of serum for test.* Since the C-F antibody appears later than the H-I antibody, the second serum specimen should be collected 3 weeks after onset of fever. This test may have special usefulness in patients from whom no serum specimen was obtained during the first week after onset of fever, because it would be possible to demonstrate development of antibody or rising antibody titer between specimens obtained shortly after defervescence and 3 weeks after onset of fever Serum specimens which are not stored in the frozen state often become anticomplementary and, therefore, unsuitable for use Sera are heated at 60° C. for 20 minutes immediately before the test.

2. *Preparation of antigen* Suckling mice are inoculated as for preparation of hemagglutinin, and their brains are harvested in the same manner. The antigens should be either benzene-extracted (i e, 20 per cent suspension in distilled water is lyophilized and extracted with benzene) or prepared by the method of Casals, using ether and acetone on frozen brains which are not lyophilized Some form of lipid extraction is necessary to avoid nonspecific reactions, which are particularly common with syphilitic sera The antigen is standardized against 16 units of

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PHLEBOTOMUS (PAPPATACI OR SANDFLY) FEVER

ALBERT B. SABIN, M.D.

Professor of Research Pediatrics, University of Cincinnati College of Medicine and The Children's Hospital Research Foundation, Cincinnati, Ohio

I INTRODUCTION

Phlebotomus (pappataci or sandfly) fever is an infectious, phlebotomus-transmitted disease of virus etiology characterized, as a rule, by fever of 2 to 4 days' duration, severe headache, pain in the eyes, generalized malaise, and leukopenia The disease is self-limited and without fatalities The only proved vector is *Phlebotomus papatasii*, and mosquitoes (*Aedes aegypti* and *Culex pipiens*) have been found incapable of transmitting the virus under optimum experimental conditions Outbreaks of phlebotomus fever are suspected, therefore, whenever an illness having the characteristics mentioned above occurs, especially among immigrants, tourists, or foreign troops in countries harboring the vector and during the hot, dry season (April to October) when the vector is most likely to be prevalent

The virus is present in the blood during the first 24 hours of the fever but the maximum amount found in the serum thus far is only 1,000 human infective doses per ml The virus has been preserved in dry ice or in the lyophilized state for approximately 9 years The disease is readily reproduced in susceptible human beings when the serum is injected intracutaneously or intravenously but only irregularly when it is injected subcutaneously or intramuscularly. Serum, infective for human beings, has been found to be without effect in a large variety of animals. The virus in human serum has been found to pass 200 mμ gradocol membranes but not membranes with an average pore diameter of 100 mμ or less The

IV. REFERENCES

1. Sabin, A. B. Chapter 33 in Rivers, T. M., *Viral and Rickettsial Infections of Man*, 2d ed Philadelphia J B Lippincott Company, pp. 556–568.
2. Sabin, A. B. Research on Dengue during World War II. *Am J Trop. Med*, 1:30–50, 1952
3. Sabin, A B The Dengue Group of Viruses and Its Family Relationships *Bact. Rev*, 14 225–232, 1950
4. Sabin, A. B, and Young, I A Complement Fixation Test for Dengue *Proc Soc Exper Biol and Med*, 69 478–480, 1948.
5. Melnick, J. L, Curnen, E. C., and Sabin, A B Accidental Laboratory Infection with Human Dengue Virus *Proc Soc Exper Biol and Med*, 68.198–200, 1948.
6. Schlesinger, R. W Propagation in Chick Embryos of the Hawaiian Strain of Dengue [illegible]
7. [illegible] *Am J Trop Med*, 1 66–77, 1952
8. Meiklejohn, G., England, B, and Lennette, E. H. Propagation of Dengue Virus in Unweaned Mice. *Am J Trop Med*, 1 51–58, 1952.
9. Sweet, B H, Chanock, R. M, and Sabin, A. B Recovery and Characterization of Hemagglutinins from Two Immunologically Distinct Types of Dengue Virus *Fed Proc*, 12 462, 1953
10. Rosen, L, Rozeboom, L E, Sweet, B H, and Sabin, A B The Transmission of Dengue by *Aedes polynesiensis* Marks *Am J. Trop. Med.*, 3 878–882, 1954
11. Sabin, A B Nature of Inherited Resistance to Viruses Affecting the Nervous System *Proc Nat Acad Sci*, 38 540–546, 1952.
12. Sweet, B H, and Sabin, A. B Development of Different Antibodies in Human Beings Experimentally Infected with Type 1 or Type 2 Dengue Viruses. *Fed. Proc.*, 13 [illegible]
13. [illegible]
14. [illegible] *J Trop Med.*, 4 198–207, 1955
15. Sweet, B H, and Sabin, A B Properties and Antigenic Relations of Hemagglutinins Associated with the Dengue Viruses *J Immunol*, 73 363–373, 1954

Intracerebral injection of the highly pathogenic, mouse-adapted sandfly fever virus produced neither clinical signs of disease nor lesions in 10 rhesus monkeys, young rabbits, guinea pigs, and hamsters Inapparent infection may have occurred, however, because they developed antibody Three human subjects were inoculated with the 10th mouse passage virus and 3 with the 20th mouse passage None became ill, but neutralizing antibodies were found in their blood 3 weeks after inoculation. Six weeks after inoculation of the mouse-adapted virus, the 6 persons were tested for active immunity by an intravenous injection of unmodified human sandfly fever virus All 6 were found to be immune, while 2 other persons who served as controls developed typical sandfly fever

Recently, the Naples strain of virus, described above, has also been adapted to 2- to 4-day-old mice. At the 35th passage it became regularly pathogenic for adult mice The identity of the mouse-adapted virus was established by neutralization tests with convalescent sera derived from persons experimentally infected with the human virus. Neutralization tests in suckling mice have confirmed the results of the cross-immunity tests in human volunteers by indicating that the Naples virus is immunologically different from the Sicilian strain

II METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

No methods of isolation are suitable for routine diagnostic purposes. To obtain a human strain of virus for research purposes, blood is drawn from suspected cases, not later than 24 hours after onset, and the serum is stored on dry ice until the clinical course of the disease in the human donors is found to be compatible with a diagnosis of phlebotomus fever, and tests in adult mice, guinea pigs, and rabbits indicate the absence of an infectious agent pathogenic for these animals The serum should then be injected intracutaneously or intravenously in doses of 1 to 2 ml. in human volunteers A strain of virus may be considered to have been recovered when a febrile illness of 1 to 4 days' duration (rarely longer) associated with leukopenia but without rash is reproduced after an incubation period usually of 3 to 7 days (may be 40 hours after intravenous injection) and further passage is possible Absolute identification of the transmissible, filtrable agent may not be possible without the demonstration that it can be transmitted by *Phlebotomus papatasii*, especially if the agent proves to be immunologically distinct from available proved strains of the virus Thus, cross-immunity tests in carefully selected

size of the virus was, therefore, estimated as being *not larger* than 40 to 60 mμ, although it was thought that it might be smaller. A strain of virus recovered during an outbreak of the disease among American troops in Naples, Italy, has been found to be immunologically different from 2 strains of virus recovered the preceding year during outbreaks in Sicily and the Middle East. It has been established that immunity to infection with homologous strains of virus can be of long duration following a single experimental attack of the disease, the longest period tested thus far being 2 years. Tests for neutralizing antibody carried out in human volunteers have yielded irregular results, suggestive evidence of neutralization having been obtained only when small amounts of virus were used.

The Sicilian strain of virus in the form of human serum, after 9 years of storage in the frozen state, was successfully adapted to the brains of newborn mice Three blind passages at 7-day intervals yielded a virus which produced encephalitis in 2- to 4-day-old mice after an incubation period of 11 to 12 days. At this stage the virus produced lesions predominantly in the hypothalamus and midbrain, but none in the muscles, fat, pancreas, or other viscera. On continued intracerebral passage in newborn mice the incubation period gradually shortened, the level of multiplication gradually rose, and after the 10th passage the virus began to kill adult as well as newborn mice. By the 25th passage in the brains of newborn mice the virus had achieved a titer of 10^{-7} (per 0 03 ml) by intracerebral injection in approximately 4-week-old mice. By gradocol membrane filtration this mouse-adapted virus was found to have a size in the range of 17 to 25 mμ.

The mouse-adapted viruses are best preserved as suspensions of brain in heated rabbit serum frozen in chests containing solid CO_2.

The identity of this highly potent mouse-adapted virus with the etiologic agent of Sicilian sandfly fever was proved by the fact that patients who had no neutralizing antibody for this virus during the acute phase of their illness, resulting from inoculation with sera brought back from Sicily in 1943, developed such antibody during convalescence Neutralizing antibodies for this virus were also found in other subjects, 4 to 5 months and even 9 years after a single experimental infection with human sandfly fever virus Persons inoculated with dengue virus developed no antibody for the mouse-adapted sandfly fever virus. The sandfly fever virus was found to be different from the dengue, West Nile, Japanese B, St Louis, and yellow fever viruses in that it regularly killed "PRI" mice, which are genetically resistant to these other viruses

PSITTACOSIS

K. F. Meyer, M.D., and B. Eddie, Dr.P.H.

The George Williams Hooper Foundation
University of California
San Francisco

human volunteers when positive can help to identify the unknown virus, but lack of cross-immunity does not signify that the virus may not belong to the phlebotomus fever group.

To recover a strain of virus in mice, it is necessary to inoculate 2- to 4-day-old mice intracerebrally with 0 01 ml. of undiluted serum. Seven to 10 days later, 5 mice are sacrificed, and a suspension of their brains is again passaged intracerebrally in suckling mice. Four to 5 blind passages may have to be carried out before an agent capable of producing a fatal encephalitis can be recovered If the recovered agent fails to produce clinical manifestations of infection in adult mice, its identity with either the Sicilian or Naples type of the virus can be established by neutralization tests with specific antisera. Since the total number of distinct immunologic types of virus is as yet unknown, negative results in neutralization tests with known sera would not eliminate the possibility that the recovered agent is a new type of phlebotomus fever virus.

An indirect way of demonstrating the presence of virus in human serum consists of inoculating 2- to 4-day-old mice intracerebrally with 0.01 ml. of serum, and 4 weeks later challenging them with 100 LD_{50} of thoroughly adapted virus which is virulent for weaned mice. A negative result in this test merely means that the serum contained less than 100 infective doses per ml, and even experimentally inoculated persons, bled within 24 hours after onset of first fever, may have less than this amount of virus in their serum

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS

Patients develop neutralizing antibodies for the homotypic virus within 2 weeks after onset of fever. Tests for antibody in acute and convalescent phase sera with the established mouse-adapted viruses can, therefore, be used to establish the presence or absence of infection with the known types of phlebotomus fever virus. Work is in progress on complement fixation and hemagglutination-inhibition tests

IV. REFERENCES

1. Sab

2 Sab

3. Sabin, A B Recent Advances in Our Knowledge of Dengue and Sandfly Fever. *Am. J Trop Med*, 4 198–207, 1955

A. Clinical Manifestations

Laboratory diagnosis is likely to be requested when the infection takes the form of an acute, influenza-like malaise differing from the more common bacterial pneumonias. The course of the untreated clinical disease is as follows

The incubation time is usually from 7 to 25 days after first contact with the agent After some vague symptoms, the acute stage begins with *general malaise, headache, and fever, succeeded by chills, vomiting,* severe headache, and lumbar pains It progresses to restlessness, insomnia, delirium, typhoidal state, with nonproductive cough and constipation The temperature rises rapidly and remains high until lysis during the 2d or 3d week. The pulse is relatively slow, an outstanding feature of the disease In late stages of fatal infections the pulse is alternately rapid and feeble, cyanosis and low blood pressure may be marked. The spleen may or may not be enlarged.

The physical signs of pulmonary involvement develop slowly and are migratory Parenchymatous consolidation begins early and affects sharply defined, irregular portions of the lobes, the lower lobe of the left lung frequently shows the primary roentgen shadows Despite extensive pulmonary involvement, sputum is scanty, mucoid, and very rarely rusty or blood-tinged

Encephalitis, bronchitis, phlebitis, thrombosis, or diarrhea may complicate the infection In some cases rose spots appear on the skin

If the patient recovers, convalescence takes weeks, is tedious, and is often interrupted by relapse Inadequate treatment with small doses of drugs administered for less than 10 days is frequently followed by serious relapses In fulminating infection the patient dies between the 10th and the 18th day of illness.

Any rapidly fatal toxemia with pneumonitis, particularly in persons more than 50 years old, deserves careful diagnostic efforts to reveal the psittacosis agent.

The clinical picture is not sufficiently pathognomonic to allow diagnosis, particularly if the disease has been treated. Nor do physical and roentgenographic examinations of the lung provide unequivocal results. The attending physician must study the illness meticulously—clinically, virologically, roentgenologically, and epidemiologically.

4 Mouse inoculation
  a. Intranasal instillation
  b Intraperitoneal injection
  c. Intracranial injection
5 Embryonated chicken egg
  a Yolk sac inoculation
  b Chorioallantoic membrane inoculation
  c Allantoic inoculation
6. Guinea pig inoculation
7. Microscopic study
  a Staining methods
    (1) Macchiavello's stain
    (2) Castaneda's stain
    (3) Giemsa's stain
      (a) Giemsa's stock staining solution
      (b) Buffered water solution according to Wilcox and Logan
  b Staining of sections
    (1) Polychromatic Giemsa stain
    (2) Noble's stain
8. Significance of the results
9 Further identification of the isolated strains
  a Pathogenicity tests
  b. Sensitivity to sulfonamides
  c. Cross-immunity tests
  d Toxin viral infectivity neutralization tests
    (1) Preparations of toxic suspensions
    (2) Diluent

E Other Diagnostic Tests
  1 Micro-agglutination test
  2. Skin sensitivity tests

III. References

I. INTRODUCTION

Psittacosis and ornithosis in man are respiratory diseases acquired by contact with a wide variety of birds. The idea that the classical form is an acute, severe, often fatal pneumonitis is being displaced by the knowledge that it is more often a subclinical disease resembling an atypical common cold. Man may acquire the infection through aerial convection (by such means as dust from soiled feathers and from dried droppings), or through contact with dead or sick birds or human beings.

the United States is the parakeet. The question of whether certain strains have become adapted to man is still open. Recently another group of strains, morphologically and serologically related to the psittacosis agents, has been isolated from the cat,[5,6] the mouse,[7,9] the calf,[10,11] the opossum,[12] and the sheep.[13,14] There is serologic evidence that some of these mammalian strains infect exposed occupational groups—slaughterers, veterinarians, and stockowners

D The Agent

The bacterium-like agents are spherical elementary bodies, 280 to 380 μ in diameter They have been called *Microbacterium multiforme*, *Rickettsia psittaci*, Levinthal-Cole-Lillie (L C L) bodies, and *Miyagawanella psittaci*, but a properly descriptive term has not yet been adopted [15] They stain purple with Giemsa, red with Macchiavello, or deep blue with Castaneda stain and are made visible through dark-field, ultraviolet, phase contrast, or electron microscopy. In smears from infected lungs, meninges, spleen, or peritoneal exudate, they form intracellular vesicles or extracellular aggregates 2 to 12 μ in diameter, scattered throughout the smear. They invade and ultimately destroy cells of the reticulo-endothelial system In man elementary bodies of diagnostic significance are rarely seen in the blood or sputum but may be visible in sections of the lung, spleen, or liver removed at autopsy In birds they are found in the air sacs and pericardial and peritoneal exudates

E Laboratory Diagnosis

Following are the minimal criteria essential for proof that a given case of pneumonitis, in any of its various gradations, is caused by a psittacosis-like agent·

1. *Demonstration of the appearance of complement-fixing antibodies* in low titer during the acute phase of the illness or of a significant rise in titer of antibodies during the period of recovery It is imperative to emphasize that early, intensive treatment with tetracycline compounds may defer or completely suppress the appearance of specific complement-fixing antibodies

2. Isolation of the agent during the acute phase of the disease, the convalescent period, or at autopsy.

B. Pathologic Lesions

In man the changes observed at autopsy are those of septicemia, with sharply defined areas of inflammation in the lungs, pharynx, larynx, and trachea, accompanied by an edematous reaction in the hilar lymph nodes, and cellular changes in the spleen, liver, and kidneys. The extent of lung involvement will depend on the stage of the illness at which death occurs. Some of the alveoli in the areas of consolidation are filled with fibrin, erythrocytes, lymphocytes, and a few polymorphonuclear leukocytes, in others the congestion and edema are replaced by desquamated or partially degenerated alveolar epithelial cells and macrophages. The bronchioles are clear or contain the same serocellular exudate as the alveoli. The mucosa of the large bronchi may be completely desquamated, favoring the secondary suppurative pulmonary complications common when patients die 20 to 30 days after onset.[1,2] In the liver, slightly enlarged and congested, there is parenchymatous degeneration; central or centrolobular focal necrotic areas are the characteristic microscopic lesions. The spleen may weigh as much as 560 gm.; it is soft and congested. The usual structural changes of an enlarged spleen are engorged sinuses filled with phagocytic cells and relatively small follicles.

The pathologic lesions in different kinds of birds dead of acute psittacosis are much alike: The pectoral muscles are wasted, and in some there is an erythematous rash and scattered macules. There are a serous purulent exudate coating the air sacs, pericardial serofibrinous exudate, and plastic deposits over the capsule of the liver. As a rule the spleen is enlarged, soft, and dark red. A swollen, engorged, and sometimes a saffron-colored, heavy liver may be studded with areas of necrosis and infarction Only rarely will lesions be found in the lungs. Chronic latent avian psittacosis is not apparent at autopsy, except for some enlargement of the spleen.[3,4]

The mammalian strains provoke a wide variety of cellular responses and are destructive to pulmonary, placental, pericardial, peritoneal, or central nervous system tissue. These are described in the articles on the isolation of these strains.[5 14]

C. Sources

ducks, egrets, and turkeys. The principal source of human infection in

the United States is the parakeet. The question of whether certain strains have become adapted to man is still open. Recently another group of strains, morphologically and serologically related to the psittacosis agents, has been isolated from the cat,[5,6] the mouse,[7,9] the calf,[10,11] the opossum,[12] and the sheep [13,14] There is serologic evidence that some of these mammalian strains infect exposed occupational groups—slaughterers, veterinarians, and stockowners

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2 Isolation of the agent during the acute phase of the disease, the convalescent period, or at autopsy.

B. Pathologic Lesions

In man the changes observed at autopsy are those of septicemia, with sharply defined areas of inflammation in the lungs, pharynx, larynx, and trachea, accompanied by an edematous reaction in the hilar lymph nodes, and cellular changes in the spleen, liver, and kidneys. The extent of lung involvement will depend on the stage of the illness at which death occurs. Some of the alveoli in the areas of consolidation are filled with fibrin, erythrocytes, lymphocytes, and a few polymorphonuclear leukocytes, in others the congestion and edema are replaced by desquamated or partially degenerated alveolar epithelial cells and macrophages. The bronchioles are clear or contain the same serocellular exudate as the alveoli. The mucosa of the large bronchi may be completely desquamated, favoring the secondary suppurative pulmonary complications common when patients die 20 to 30 days after onset.[1,2] In the liver, slightly enlarged and congested, there is parenchymatous degeneration; central or *centrolobular focal necrotic areas are the characteristic microscopic* lesions. The spleen may weigh as much as 560 gm.; it is soft and congested. The usual structural changes of an enlarged spleen are engorged sinuses filled with phagocytic cells and relatively small follicles.

The pathologic lesions in different kinds of birds dead of acute psittacosis are much alike. The pectoral muscles are wasted, and in some there is an erythematous rash and scattered macules. There are a serous purulent exudate coating the air sacs, pericardial serofibrinous exudate, and plastic deposits over the capsule of the liver. As a rule the spleen is enlarged, soft, and dark red. A swollen, engorged, and sometimes a saffron-colored, heavy liver may be studded with areas of necrosis and infarction Only rarely will lesions be found in the lungs. Chronic latent avian psittacosis is not apparent at autopsy, except for some enlargement of the spleen.[3,4]

The mammalian strains provoke a wide variety of cellular responses and are destructive to pulmonary, placental, pericardial, peritoneal, or central nervous system tissue. These are described in the articles on the isolation of these strains.[5-14]

C. Sources

The agent of psittacosis or ornithosis has been found in 72 species of birds,[3] including psittacine birds, particularly parakeets, panama parrots, canaries, finches, linnets, fulmars, sea gulls, pigeons, doves, chickens, ducks, egrets, and turkeys. The principal source of human infection in

duced in natural infections. Experimental immunization incites elaboration of additional specific toxin- and infection-neutralizing antibodies in readily demonstrable quantities. The heat-stable and heat-labile antigens can be detected by complement fixation tests and differentiated by absorption.

At present the direct complement fixation test of at least two serum samples, one collected during the acute and one during the convalescent phase, is the means of making the earliest possible serologic diagnosis of human infections by a member of the psittacosis group. It is also useful in surveying flocks of pigeons and shipments of imported psittacine birds. Since the serum of latently infected birds fixes complement, this test is the best tool for quickly detecting possible sources of infection.

The test was developed by Bedson and his colleagues[10,19-21] and has been extensively applied at the Hooper Foundation.[22] The sera of man, guinea pigs, psittacine birds, pigeons, doves, monkeys, sheep, cattle, and probably other animals infected with these agents fix complement in the presence of their common antigen. The preparation of adequate antigens suitable for the test has been difficult. Antigens prepared from cultures grown on Rivers-Li medium or Zinsser's solid medium,[23] and from allantoic fluid from infected fertile eggs, yolk sac or mouse lung by centrifugation and boiling[24] or by treatment with phenol have all been used. Heating or addition of phenol, or both, destroys all but the group antigen. The technical procedures are now standardized to the use of boiled and phenol-treated yolk sac antigens. Such an antigen provides a *group* reaction, not a specific strain reaction. Recent observations indicate that some sera derived from infected parakeets, pigeons, and other avian species may not fix complement in the presence of crude or even purified yolk sac antigens. By substituting antigens prepared from mouse lungs, the appropriate reactions may be obtained in the direct test. The general pattern of reaction by bird sera in the complement fixation system is variable and must be evaluated with different antigens and in the direct and indirect test.[24a]

It is possible, by absorbing a psittacosis serum with the group antigen, to remove the group antibody and to leave the specific one (p. 410). A complement fixation test then made with the absorbed serum and fresh untreated viral agents possessing the specific labile antigen in a reactive state becomes specific, and a certain distinction between a psittacosis and lymphogranuloma venereum agent can be made.[25] The preparation

II. ROUTINE LABORATORY PROCEDURES

A. Precautions

Specimens suspected of containing the agent of psittacosis must be regarded as highly pathogenic and dangerous. The extreme infectivity of these agents for man does not recommend the examination of such material in the average public health or clinical laboratory. Well-trained, experienced workers have contracted psittacosis, even when working in specially equipped laboratories under carefully controlled conditions.

The scattering of infected material through desiccated fecal droppings adhering to the down and feather particles of psittacosis-infected birds is especially dangerous The manipulations required in preparing antigens for serologic tests are equally so, particularly the pipetting and centrifuging of suspensions of viral elements; a special, isolated laboratory room should be reserved for these practices. All manipulations should be conducted under a hood in which the air is treated with ultraviolet lamps or is suitably exhausted The liberal use of aerosols, such as propylene glycol, in the rooms is recommended. Technicians should wear special gowns, rubber gloves, suitable masks, and head covers to protect their hair. After use, all clothing should be placed in a cloth container and immersed in hot, strong soap solution, or steamed The worker should wash his hands and face in hot, soapy water or take a shower when his work is finished

A separate insect-proof and dust-proof room is essential for housing experimentally infected birds and animals; the floor of the room should be treated regularly with dust-fixing oils, and the door and window sashes must be sealed with special plastic material to prevent dust from escaping into halls and adjacent rooms This room should be restricted to the virologist and one well-indoctrinated animal caretaker

Those in charge of psittacosis studies should keep a watchful eye on the health of the laboratory personnel, bearing in mind that those who are treated early and intensively with effective antibiotics may become reinfected

B. Direct Complement Fixation Test

It is important in understanding the basis for this and some of the other diagnostic tests to know something of the antigenic composition

or even to autoclaving at 135° C.,[17] are demonstrable. The labile component is destroyed by phenol, hydrochloric acid, or papain.[17,18] The heat-stabile antigen resists proteolytic enzymes, but not potassium periodate, even at low concentrations; this behavior suggests that this antigen is a carbohydrate.[17] Antibodies to these two antigens are pro-

on Adherence to Conventional Technic in the Performance of Reliable Serologic Tests for Syphilis should be followed in the preparation of sheep blood cells, hemolysin, and complement.

a. Yolk sac antigen. Preparation of the other types of antigens is described in the previous edition,[27] but since in recent years the yolk sac antigen has been adopted as a standard antigen in all diagnostic laboratories,[39] only the antigens which are in use at the George Williams Hooper Foundation will be dealt with here.

Inoculate 7-day-old embryonated eggs directly into the yolk sac. The suspension of the adapted psittacosis virus to be used is standardized so that the majority of the embryos die between the 56th and 72d hour after inoculation with the 0 25 ml. of the dilution of 10^{-4} This requires a virus which is so adapted that a dilution 10^{-8} to 10^{-10} kills the embryos. Harvest the yolk sac as soon as possible after death of embryos and examine them for elementary bodies, if rich in virus, pool and weigh. Grind thoroughly in a mortar with sterile sand and add beef heart broth pH 7.0 to make a 20 per cent suspension (A Waring blendor presents risks) Culture for sterility Hold in the refrigerator over a period of 3, preferably 6, weeks The emulsion is then lightly centrifuged to remove coarse particles and put in a heavy, sterile pyrex flask, which is steamed in an Arnold sterilizer at 100° C or immersed in boiling water for 30 minutes When cool add to the heated emulsion liquefied phenol to a concentration of 0 5 per cent Permit the antigen to ripen in the refrigerator for at least one week If the emulsion is kept sterile the antigenicity will be maintained for at least one year

Improvement in the purification of yolk sac antigen appears to be possible in the light of studies by Nigg, Hilleman, and Bowser[28] on lymphogranuloma venereum preparations, and those of Whitney and Gnesh [29]

Before use in the complement fixation test it is titrated in 2-fold serial dilutions against a constant amount of human or pigeon serum containing known specific antibodies against the psittacosis agent In the actual test it is diluted so that 0 25 ml contains 4 complement-fixing units. The box-titration technics as used by Kolmer are quite satisfactory

3. *Pretesting of complement.* If one has an unknown antigen and no titered antigen to use as standard, it is necessary to pretest the complement before it is titrated because the approximate units of antigen have to be used in the complement fixation test before the unit of complement can be determined Make an arbitrary dilution of antigen; 1.4 is a safe

of suitable specific antigens in adequate amounts for use in the absorption test, however, is difficult.

One point must be made clear: only an antigen prepared from a known psittacosis strain should be used. Any antigen, experimental or commercial, prepared with a lymphogranuloma venereum agent is not satisfactory for the serodiagnosis of psittacosis.

Persistence of complement-fixing antibodies after recovery from infection, anamnestic reactions, cross reactions with other members of the psittacosis group and with the lymphogranuloma venereum agent, chronic psittacosis infections, or reinfection and effective treatment must all be considered in interpreting the results of this test

1. *Diagnostic materials*

a Human serum. Blood should be collected first on the day that the patient with symptoms of pneumonitis suggestive of psittacosis is examined, and thereafter on the 8th, 16th, 30th, and 40th days—that is, throughout the illness and convalescence The blood sample (10 to 15 ml.) must be collected aseptically from the vein and allowed to clot. If it is to be shipped any distance, the serum must be removed aseptically from the clot and sent by air mail It should not be frozen.

b. Bird serum. Blood samples of the larger birds (pigeons, parrots, cockatoos, conures, macaws, doves, turkeys, egrets, ducks, or others) can be obtained easily with a 2 to 5 ml. syringe and a 24-gauge needle The blood is usually taken from the wing vein. Parakeets may be bled from the jugular vein or heart To enable an assistant to hold the bird more easily while the blood is drawn, Amazon parrots, cockatoos, macaws, and other birds of similar size may be put under light anesthesia.[23]

If the blood is put into tubes first prepared with a very thin coating of vaseline or embedding paraffin, evenly applied, the yield of clear serum will be greater For shipping, carefully withdraw the serum into the prepared tube, cork tightly, label, and send by air express

c. Other mammalian serum. Puncturing the jugular vein is the most satisfactory means of obtaining blood samples from sheep, cattle, or goats. The radial or saphenous vein is chosen in the dog or cat. The handling of the blood is the same as described above.

2. *Reagents* include antigens, proven positive serum, guinea pig complement, rabbit antisheep hemolysins, and sheep blood cells. Titration of the antigen is preceded by the preparation of the hemolytic system and its adjustment. Kolmer's technic [28] approved by the Committee

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dilution because the actual unit is usually high. Sera showing an equal degree of inhibition of hemolysis in the first 3 tubes carrying antigens and in the last 3 tubes carrying no antigens may be used but will usually be found to have a low unit of activity. Any serum showing a greater degree of inhibition of hemolysis in the first 3 tubes carrying antigens than in the last 3 tubes carrying no antigens should be regarded unsatisfactory for the test so far as the particular antigen is concerned.

The final complement titration determines the smallest amount of complement giving complete sparkling hemolysis. This amount is the exact unit. The next higher tube is the full unit, which contains 0.05 ml. more of complement. For example, if the exact unit is 0.15 ml., 1 full unit is 0 2 ml and 2 full units is 0.4 ml.

It is not necessary to repeat the hemolytic and anticomplementary titrations of a given lot of antigen after it has been established, but it is desirable to titrate it occasionally for antigenic activity.

4. *The test.* The procedure is summarized in Table 1.

5. *Interpretation.* If the results of the complement fixation test are to be considered at all indicative, their behavior during the course of the infection must be followed. It is almost impossible to draw sound diagnostic conclusions on the basis of a test of one serum specimen. Characteristically the antibody level continues to rise during the acute illness to a peak during convalescence. The level gradually declines thereafter, sometimes over a period of years, depending on the stimulus from persisting organisms The level may rise slightly in the acute stages of other infectious diseases, but it will decline rather than rise during convalescence If the effective antibiotics are given in large quantity early in the i

[illegible]

serologic evidence of psittacosis; the two samples should be tested in the same test. A titer of 1.16 ++++ or higher in a single serum sample after the 1st week of illness is highly suggestive. The titer by the 14th day is usually 1:32 ++++ or higher. If the titer has reached a high level by the time the first sample was taken, it may remain at the same level during convalescence.

A titer of 1·8 ++++ in a single serum sample indicates that the patient has had previous experience with a psittacosis agent and is now having an anamnestic reaction, that he is now in the early stages of acute psittacosis, or that he has lymphogranuloma venereum If there is any

TABLE 1

THE ACTUAL COMPLEMENT FIXATION TEST *

Tube Number	Patient's Serum in Twofold Dilution, 0 25 ml	Antigen (4 units), ml	Complement (2 full units), ml		Saline	Sensitized Cells, ml	
1	1 2	0 25	0 5	Water bath for 2 hours, 37°C		0 5	Water bath for ½ hour, 37°C, after controls clear
2	1 4	0 25	0 5			0 5	
3	1 8	0 25	0 5			0 5	
4	1 16	0 25	0 5			0 5	
5	1.32	0 25	0 5			0 5	
6	1 64	0 25	0 5			0 5	
7	1 128	0 25	0 5			0 5	
8	1 256	0 25	0 5			0 5	
Anticomplementary Control on Each Serum to be Tested							
9	1.2	0	0 5		0 25	0 5	
10	1 4	0	0 5		0 25	0 5	
11	1.8	0	0 5		0 25	0 5	
12	1 16	0	0 5		0 25	0 5	
System Control, 1 Set for a Run							
13	0	0 25	0 5		0 25	0 5	
14	0	0	0 5		0 50	0 5	
15	0	0	0		1 00	0 5	

* Test serums heated at 56° C for 30 minutes before diluting

doubt about the latter the Frei test should be used In persons constantly exposed to psittacosis agents—aviary owners, petshop employees, pigeon breeders, and others—titers will range from 1 8 to 1.32 ++++ in the absence of acute infection

Titers of 1:4 ++++ and higher in the sera of psittacine birds or pigeons indicate either latency of or recovery from infection Active shedders usually have high titers, pigeons with "closed" latent infections may have no complement-fixing antibodies It may be impossible to isolate the agent from birds with high titers

6. Bedson's method of absorption of sera.

To improve the specificity of the complement fixation test and to distinguish psittacosis from lymphogranuloma infection, Bedson[30] has proposed a special absorption test.

a Absorption of the agent. Suspensions of the spleens of mice infected with the psittacosis agent are partially purified by centrifuging in a horizontal centrifuge at 1,000 to 2,000 r p.m. for 5 minutes, and the supernatant is then centrifuged in an angle centrifuge at 5,000 r p.m. for 2 hours The pellet deposit is suspended to volume in saline and steamed for 20 minutes (Yolk sac or allantoic grown agents can be used, but the elements are frequently more difficult to remove entirely from the absorbed serum)

b. Absorption of serum. The yield from 1 ml. of suspension is required for absorption of 1 ml. of serum suitably diluted (the dilution should leave the antibodies in a concentration of 8 times the titer). The required amount of steamed suspension is centrifuged in an angle centrifuge at 3,000 r p m. for 1 hour, the supernatant is discarded, and the deposit taken up to volume in the diluted serum. Mix thoroughly and hold in a refrigerator overnight Centrifuge at 5,000 r p.m. in an angle centrifuge for 2 hours and use the supernatant absorbed serum in the complement fixation test with fresh unheated virus as antigen.

c. *Results* The fixing power of the serum is abolished or very considerably reduced for steamed homologous or heterologous virus and for fresh heterologous virus The major part of antibody for the fresh homologous virus is left untouched

For the study of unidentified strains the value of sera absorbed with heated strains is unquestionable, but the procedure is hardly suitable for routine use because the risk with live antigens is too great. Moreover, the amount of specific antigen in freshly made suspensions of unheated active agents is unpredictable The results of Sigel's experiments[31] indicate that the group reactive substance is not a homogeneous antigen, but consists of at least two constituents.

C. Complement Fixation Inhibition (Indirect) Test

The sera of chickens, ducks, turkeys, pheasants, some pigeons, and other birds infected with the psittacosis or ornithosis virus do not fix complement in the complement fixation system in the presence of specific antigen.[24a,32,33] The inhibiting antibody lacks some character that would enable it to combine with guinea pig complement. This lack is not the same as that in the incomplete antibody, since bird sera can give agglu-

tination in the presence of suitable antigen. The inhibitory antibody in the sera of chickens or ducks immunized or infected with a psittacosis agent combines with the specific antigen without fixing complement in the indirect complement fixation test. The reaction is revealed by adding specific complement-fixing indicator antibody (serum from naturally infected pigeons or man, or from animals immunized) to the chicken serum-antigen mixtures; if all the antigens have been used to saturate the inhibiting substance in the chicken serum, then none is left to combine with complement-fixing antibody and complement, yielding hemolysis.

1. *Titration of indicator antibody serum.* This is the same as was described under the direct complement fixation test. The complement and hemolytic systems are titrated in the usual manner. Sera of naturally infected pigeons have proved most active as a source of indicator antibody for this test. The pigeon serum is inactivated at 56° C. for 30 minutes and distributed in 0.25 ml. amounts of serial 2-fold dilutions to three series of tubes. Add 0.25 ml. of antigen to each series: 1st series, 0.25 ml. containing 4 units of antigen; 2d series, 0.25 ml. containing 2 units of antigen; 3d series, 0.25 ml. containing 1 unit of antigen. To all tubes add 2 exact units of complement, shake thoroughly, incubate the mixtures in a water bath at 37° C. for 2 hours and then add 0.5 ml. of the hemolytic system to each tube. Place in water bath 37° C. for 1 hour. The technic and results of a sample titration are shown in Table 2.

TABLE 2

TITRATION OF PIGEON SERUM FOR SELECTION OF THE PROPER DILUTION FOR TEST

Pigeon Serum Dilutions, 0.25 ml	0.25 ml Antigen Units			Complement 2 Exact Units, ml		Sensitized Cell, ml	
	4	2	1				
1:32	++++	++++	+++	0.5	Water bath for 2 hours, 37°C	0.5	Water bath for 1 hour, 37°C
1:64	++++	++++	++	0.5		0.5	
1:96	++++	++++	++	0.5		0.5	
1:128	++++	++++*	++	0.5		0.5	
1:256	+++	++	±	0.5		0.5	

* The highest dilution of serum with the smallest amount of antigen showing a ++++ is the dilution to use in the test, which would be 1:128.

2. *Procedure of the test.* Make 2-fold dilutions of the inactivated (56° C. for 30 minutes) serum to be tested, starting with 1:2. Mix 0.25 ml. of diluted test serum with 0.25 ml. of the titrated pigeon serum; add antigen (1 unit) and complement (2 exact units). Incubate the mixture at 37° C. for 2 hours. Add 0.5 ml. of sensitized sheep blood cells. Incubate the mixture at 37° C. for 1 hour. The procedure and results of an indirect complement fixation test are shown in Table 3.

3. *Results.* The degree of complement fixation is recorded in the usual way: ++++ (complete fixation) to ± (little fixation). The interpretation of the results is directly opposite that of the direct test: complete hemolysis (no fixation) indicates a positive result. The chicken serum in the sample test in Table 3 has an inhibition titer of 1:8 and this titer is considered diagnostically significant for this serum. Titers have reached 1:64 or higher with sera of naturally infected ducks and turkeys.

D. Isolation of the Agent

1. *Collection and preparation of diagnostic materials. Blood* is collected during the 1st week of illness, *before* treatment is instituted, or during a relapse, and is defibrinated or clotted. The defibrinated blood or the clot, but not the serum, must be frozen as soon as possible after it is collected. *Sputum* is the most likely source of the agent, particularly in the early stages of the disease, but unfortunately it is often scanty or unobtainable. Every effort should be made to get a specimen, however small, with the aid of expectorants if necessary. It should be promptly frozen in a sealed container and sent in the frozen state to the laboratory. *Pleural fluid* must be taken aseptically, placed in a sterile tube, and sealed with a rubber stopper. The amount obtainable may vary from 1 to 5 ml.; it may be shipped if frozen and packed in dry ice. *Vomitus* must be collected in a sterile basin and transferred to the laboratory at once. It cannot be shipped satisfactorily. Specimens from the throat are obtained from garglings or by washing the patient's pharynx with sterile saline (10 to 20 ml.). If the material is to be shipped any distance for testing or if the test must be delayed several hours, it should be kept frozen until used.

At autopsy, obtain any pneumonic part of the lung, a small section of the spleen and liver, and pleural or pericardial effusions if they are present. Deposit small pieces of tissues in sterile bottles or dishes and take at once to the testing center; there they should be frozen if not used within approximately 5 hours. Tissue sections to be sent any appreciable

TABLE 3

The Complement Fixation Inhibition Test *

Tube Number	0 25 ml of Test Serum Dilutions	Pigeon Serum†	Antigen, 1 Unit	Complement (2 Exact Units)		Sensitized Cells (2 Units Hemolysin) (2 Per Cent Sheep Cells)		Example Readings
		ml.	ml	ml		ml		
1	1 2	0 25	0 25	0 5	Shake well, water bath, 37°C for 2 hours or icebox 6°-8°C for 9 12 hours followed by 10 minutes, 37°C before adding cells.	0 5	Shake well, Water bath, 37°C for 1 hour	0
2	1 4	0 25	0 25	0 5		0 5		0
3	1 8	0 25	0 25	0 5		0 5		0
4	1 16	0 25	0 25	0 5		0 5		++
5	1 32	0 25	0 25	0 5		0 5		++++
6	1 64	0 25	0,25	0 5		0 5		++++
7	1 128	0 25	0 25	0 5		0 5		++++
8	1 256	0 25	0 25	0 5		0 5		++++

Controls

Tube Number	0 25 ml of Test Serum Dilutions	Pigeon Serum†	Antigen 1 Unit	Complement (2 Exact Units)	Buffer Saline		Sensitized Cells (2 Units Hemolysin) (2 Per Cent Sheep Cells)		Example Readings
9	1 2 serum	0	0 25	0 5	0 25	Water bath, 37°C. for 2 hours	0 5	Water bath, 37°C for 1 hour	0
10	1 2	0	0	0 5	0 5		0 5 (If sera is AC would depend on degree as to +, ++, +++, or ++++)		0
11	1 4	0	0	0 5	0 5		0 5		0
12	1 8 lysin	0	0	0 5	0 5		0 5		0
13	1 2 pigeon serum	0	0	0 5	1 0		0 25 2 per cent cells only		++++
14	0	0 25 of dilution used in test	0 25 2 units	0 5	0 25		0 5		++++
15	0	0 25 of dilution used in in test	0 25 1 unit	0 5	0 25		0 5		++
16	0	0 25 of next 2 fold dilution	0 25 2 units	0 5	0 25		0 5		++
17	0	0 25 of same dilution as in 16	0 25 1 unit	0 5	0 25		0 5		±

* A known positive and a known negative serum must be included in each run of the test Reading the test—the titer is the highest dilution of serum which still gives complete or nearly complete inhibition For example, the reading in the above test would be 1 8 and partial in 1 16

† The pigeon serum dilution to be used in the test is 0 25 ml of that dilution which gives a ++++ reaction with the smallest amount of antigen. See Table 2

distance should be wrapped in sterile gauze and placed in waxed envelopes or foil paper. Put in a light metal container (coffee cans are excellent), seal tightly, freeze, pack in dry ice, and ship by air express. Tissues to be examined microscopically must be fixed, preferably in Schaudinn's solution, and not frozen.

In the course of an epidemiologic survey single birds are usually submitted for autopsy and isolation of the agent. To prepare the body for shipping, wrap it in cheesecloth previously soaked in a 2 per cent lysol solution, put it in a container, seal and freeze if possible, and ship it packed in dry ice.

If a number of bird flocks are being tested for latent infection, a 10 per cent sample of each flock is sacrificed. This is best accomplished by asphyxiation with coal gas or chloroform. To protect the personnel of shipping companies, never attempt to ship living birds suspected of infection except under special instructions of a health officer.

a Preservation of the virus in the laboratory. Specimens under test, including field specimens, may be held in lusteroid tubes, glass vials, or other tightly sealed containers Freeze specimens at –70° C. and keep frozen until used for animal inoculation. Such material may be stored safely from 1 week to several months, preferably not longer because infectivity is gradually lost, especially if the material is not sterile.

If fairly clean, the material may be held for several days in the ordinary refrigerator (temperature 30° to 40° F.) without noteworthy loss in potency of the agent Organs of mice or birds heavily infected may be kept frozen for at least 4 years without noticeable effect. Egg material or organ emulsions lose infectivity within 3 to 4 months. Strains are best preserved for long periods by desiccation from the frozen state (lyophilization). Store them *in vacuo* in hermetically sealed tubes, in the refrigerator Under such conditions the agent remains alive at least 4 years.

Glycerol inactivates the agent.

b. Preparation of materials for animal inoculation

(1) Blood Thaw out the sample and inoculate undiluted intraperitoneally and intranasally into mice.

(2) Tissues. Frozen tissue is first slowly thawed in a refrigerator at about 0° C.; this usually requires 18 to 24 hours. Weighed portions held in petri dishes are minced with sterile scissors and then transferred to a sterile mortar or grinder (tissue grinder A.H.T. specification 4288-B), where they are ground to a paste with sterile sand, glass particles, or carborundum (size 60) A very convenient grinder is a 150 x 20 mm

pyrex test tube in which a narrower, but longer and stouter, test tube (200 x 10 mm.), with a roughened outer surface, acts as a pestle There is less risk of contamination with this device than with a mortar. Large pieces of organs are minced in a special metal container which can be hermetically closed and operated on a Waring blendor base (Catalogue No 174246, Central Scientific Company)

After the tissue has been ground thoroughly, the volume of diluent required to make a 10 to 20 per cent emulsion is run into the tube, and the whole is thoroughly mixed Employ as diluent plain hormone broth, pH 7.4-7 6, or if material is contaminated use TSS (p 416) If no particular urgency exists it is best to hold all suspensions in the refrigerator for 18 to 24 hours because this allows additional sedimentation and diffusion of the agent in the diluent

A sample of the emulsion is cultured to detect possible bacterial contamination before it is put in the refrigerator Should it be grossly contaminated the bacteria must be partially or completely removed before inoculation in the appropriate way (p 416)

(3) Sputum or throat garglings Sputum is thawed and cultured for bacteria on blood agar plates To prepare the emulsion, suspend the sputum, depending on its consistency, in 2 to 10 times its volume of sterile hormone broth (pH 7 2 to 7 4), emulsify by thoroughly shaking with glass beads in a sterile, carefully stoppered container Refrigerate the material for 18 to 24 hours at about 0° C for extraction of the agent

Centrifuge the extracts for 20 to 30 minutes at 3,000 r p m When the bacterial flora of the sputum sample consist of large numbers of beta streptococci or pneumococci, treat the emulsion with antibiotics Prolonged contact of specimens with such drugs must be avoided, because the psittacosis agents are susceptible to their action

(4) Pleural fluid or vomitus Determine the extent of bacterial contamination in pleural fluid by culturing on blood plates When the contaminants have been properly dealt with, hold the specimen in the refrigerator (about 0° C) until inoculation. Vomitus is also cultured on blood plates to learn the type of contaminant, and when the sample is heavily contaminated it is best treated with antibiotics When a large quantity of vomitus is available the coarse material is sedimented by centrifugation, and the supernatant is either filtered (Berkefeld V) or the viral elements are deposited by prolonged high-speed centrifugation (15,000 r p m.). Since the agent is present in low concentration, mice should be repeatedly injected with the filtrate on consecutive days.

2. *Treatment of contaminated materials.* Diagnostic materials may be decontaminated in any of the following four ways: (1) For moderate contamination, light centrifugation (about 5 minutes at 3,000 r.p.m.) may suffice. (2) Coarse filtration through sand and paper filters may be used (3) Centrifugate or coarse filtrates may be passed through an Elford-type gradocol membrane having an average pore size of 1.0 μ. This final filtrate is injected intraperitoneally into mice in at least 1.0 ml. quantity. (4) Since there is always a definite, and sometimes a complete, loss of the agent through filtration, the use of bacteriostatic agents (antibiotics) is preferred. On the basis of observations by Morgan and Wiseman[34] and Wiseman and associates,[41] the following stock solution (TSS solution) is recommended:

Tyrothricin, 25 mg per ml.	0.08	ml
Sodium sulfadiazine	50	mg
Streptomycin hydrochloride	25	mg (250 units)
Beef heart broth, pH 7.0 to 7.6	100	ml

Dissolve 50 mg of sodium sulfadiazine in 100 ml. of sterile broth. Autoclave the mixture for 30 minutes at 15 pounds and cool. Add the streptomycin and tyrothricin, cap, and store in the refrigerator. This mixture of antibiotics if used judiciously in short exposure will not injure the virus.

3. *Isolation.* Elementary bodies may be found directly in impression preparations of the air sac, in pericardial or peritoneal exudates of birds, or in sections of properly fixed, but not frozen, tissues (lung, spleen, or liver) excised at autopsy in man They will rarely be found in the blood, sputum, or other excretions of man, so that it is usually necessary first to increase the amount of the agent by inoculation of 4 to 6 mice or 12 eggs and then to search for them in the exudates or gross lesions of the diseased tissues. This is achieved by intranasal instillation and intraperitoneal or intracranial injection of mice with the emulsion being tested or by yolk sac inoculation of 7-day embryonated eggs. Unfortunately, spontaneous, latent, psittacosis-like infection[7,9,35] in the lungs is a constant and vexing complication of mouse inoculation. The presence of murine pneumonitis virus easily leads to false conclusions, this cannot be overemphasized. For isolation of the mammalian strains, intraperitoneal injection of guinea pigs is the method of choice.

4. *Mouse inoculation.*

a. Intranasal instillation Instill 0.03 to 0.05 ml of a 10 per cent

tissue suspension, with the mouse in light anesthesia induced with ether or barbiturate.

If the material inoculated is virulent, signs of infection develop rapidly: hunched posture, apathy, and increasingly labored respiration. Death follows within 48 hours to 20 days The early deaths are due to toxin With less virulent material, recovery may occur, accompanied by gradual disappearance of all symptoms.

Segments or entire lobes of the lung may be extensively consolidated Death may be produced between the 8th and the 16th day if the agent is present in dilutions of 10^{-4} Discrete foci of pneumonia are manifested as the limiting infective dilutions are approached. These areas are gray, almost translucent, 1 to 3 mm. in diameter, and lie in apparently normal lung

Elementary bodies become progressively less numerous in smears made from lungs in which the infection has been established for more than 10 days, and one may encounter great difficulty in finding them in old lesions where repassage may furnish excellent material for microscopic examination.

To isolate strains from pneumonic lesions in kittens and sheep or in the central nervous system of opossums,[12] it is customary to inoculate mice intranasally with 10 per cent emulsions of the affected tissue If the agent is present, definite pneumonia with more than half of the lung substance consolidated is noted when the mice are sacrificed on the 10th day Serial passage increases the virulence, and death ensuing 2 to 3 days after inoculation becomes fairly constant.[5,6]

b Intraperitoneal injection. Although most psittacine, turkey, and egret strains will be revealed by inoculation by this route, those from pigeons, chickens, or ducks may produce significantly enlarged spleens and cause death only irregularly.

Administer 0.5 ml of the prepared 10 or 20 per cent sterile emulsion. Virulent material from parrots, parakeets, man, and, occasionally, canaries, when injected by this route in this amount causes death of the mouse in from 3 to 30 days The average infection lasts 3 to 10 days A few animals recover A short incubation period after injection of avian material is indicative of a highly virulent and toxic strain, such as the turkey or egret strains, present in the bird in high concentration

If the mice die within 2 to 3 days, little that is abnormal can be seen with the naked eye; the spleen and liver may appear normal in size and architecture *Quite characteristic, and often the only sign at this*

time, is the bloated duodenum covered with a thin viscous exudate, and its content consists of only a little chyme. In some animals the surface of the liver and the intestines may be moist and covered with a thin, sticky exudate which generally contains abundant endothelial cells packed with viral particles. When death occurs within 5 to 15 days the spleen is enlarged, and early necrotic lesions of the liver are visible to the naked eye. Microscopically, hemorrhages and necrosis are common in the liver; the cells of both the liver and spleen are packed with viral bodies.[36] Sometimes the abdominal cavity is filled with stringy, turbid, fibrinous exudate.

Mice that recover and are sacrificed many weeks after infection have few lesions. In general, the intestines are slightly distended and light colored. Exudate may be present in the abdominal cavity. The spleen is conspicuously enlarged and the liver friable and mottled. The kidneys are grayish. Although it is difficult to find the viral bodies in the tissue smear, passage inoculations have demonstrated that they may exist as long as 300 days after the initial infection. Mice that recover ordinarily possess an infection immunity.

If the animals survive to the 30th day, it is imperative that they be sacrificed and further passage of emulsions of their spleens and livers be made in other mice. At least 3 such passages are necessary before a negative report can be made.

c Intracranial injection. Sterile exudates from the pericardial or air sacs of birds or peritoneal fluid and suspensions prepared from infected mice or guinea pigs may be safely injected by this route. This sometimes furnishes excellent histologic specimens for rapid diagnosis. Inoculate 0.03 to 0.05 ml. of a 10 per cent emulsion. Somnolence and paralysis often develop within 24 to 48 hours, followed by death within 3 to 5 days. This route has the advantage that it excludes the respiratory tract and thus precludes the possibility of accidentally activating a latent mouse pneumonitis.

5. *Embryonated chicken egg.*

a. Yolk sac inoculation. This simple and direct method is ideal for enriching growth of the agent and for producing large quantities of infectious material with little contamination.

Six to 12 eggs are thoroughly cleansed with Roccal diluted 1:70,000 and then are candled. The air sac and yolk sac are marked. Using a syringe with a 20-gauge needle 1½ inches long, inject 0.5 ml. of a broth suspension directly into the yolk sac of a 6- or 7-day embryonated egg. Cover

the needle puncture with a paraffin-beeswax mixture and incubate the egg at 37° C Candle the eggs each day When the embryo is becoming enfeebled, it should be watched closely by candling several times a day; it usually dies in 3 to 8 days. After the shell of the egg has been carefully disinfected, it is opened with a sterile forceps and scissors. Open the top of the egg, expose the yolk sac, and aspirate the yolk material with a sterile large-gauge needle Carefully lift the yolk sac from the egg with sterile forceps and place it in a sterile petri dish. It should be washed with care in sterile saline until free of yolk material Impression smears are made and stained to determine whether or not elementary bodies are present, microscopic lesions have not been noted after yolk sac inoculation

b Chorioallantoic membrane inoculation[37] This is primarily an experimental method used to study the life cycle of these agents Inoculate 0 05 ml of the emulsion to be tested directly into the chorioallantoic membrane of a 10- to 12-day embryo Distinct poxlike lesions appear on the membrane in from 3 to 6 days, followed by death of the embryo

c Allantoic inoculation This is the best method of obtaining concentrated solutions of pure elementary bodies free of yolk material The egg is candled, and the air sac and allontoic sac are marked. The 9-day-old embryo is preferred Disinfect the shell of the egg and inject 0 25 ml. of yolk-sac membrane suspension or 0 25 ml of undiluted allantoic fluid directly into the allantoic sac The dilution most suitable for the inoculum is that which will kill the embryo in 4 days Each egg yields from 5 to 10 ml of clear allantoic fluid Chilling the eggs overnight in the refrigerator before harvesting the agent increases the yield The resultant preparation consists almost entirely of elementary bodies, with very little extraneous material

6 *Guinea pig inoculation* Intraperitoneal injection of mammalian strains results in febrile illness frequently terminating fatally in 5 to 10 days The infection is characterized at autopsy by fibrinous peritonitis with effusion of serosanguineous fluid into the peritoneal and pleural cavities Whenever mammalian tissues are examined for a latent infection, series of mice should also be inoculated by various routes with the emulsions properly freed from bacterial contaminants

Most avian strains, except those from egrets or turkeys, are avirulent for guinea pigs All strains induce fever, with temperatures as high as 104° F, lasting for 2 to 4 days The febrile reaction varies from animal to animal and may be followed by emaciation. Most animals injected

with psittacine, pigeon, duck, or chicken strains recover, but occasionally one will die. The serum of guinea pigs that recover contains specific antibodies. Passage through guinea pigs does not diminish the pathogenicity for mice, ricebirds, or parrots.

Most avian and mammalian strains bring about pneumonic lesions involving parts of one or more lobes of the lung on intranasal inoculation of 0 02 to 0 05 ml. of organ suspensions. Death follows within 4 to 10 days.

7. *Microscopic study* The staining reactions and morphologic appearance of these agents are so characteristic that identification in smears from the infected tissue of the bird, mouse, or other mammals is usually easy. In tissues of spontaneously fatal infections of birds and of experimentally infected mice and birds that die of rapidly fulminating disease, the colonies may be seen in all different stages of development. Some experience is no doubt necessary in recognizing and differentiating the organisms from other cytoplasmic granules situated in the exudates and tissues and from the fat globules in the yolk sac preparations, but with the aid of the Macchiavello stain, the bright red elementary bodies should be readily identified, even by the beginner.

a. Staining methods

(1) Macchiavello's stain Prepare the following stock solutions.

Basic fuchsin ..	. 0.25 gm	in 100 ml of double distilled water
Citric acid . .	. 1 gm	in 200 ml of double distilled water
Methylene blue	1 gm	in 100 ml of double distilled water

After drying in air, the smear or the impression preparation is fixed by heat. The basic fuchsin solution, first passed through filter paper in a small funnel, is dropped onto the film and allowed to remain for 5 minutes The fuchsin is then drained off, and the slide quickly dipped for a few seconds into the citric acid solution, which is best held in a Coplin jar Wash the slide thoroughly with tap water and stain with 1 per cent methylene blue for 20 to 30 seconds, wash again in tap water, and dry by blotting Long exposure to citric acid will decolorize the elementary bodies, and they will all stain blue In a properly prepared slide, most of the viral bodies will be stained red

(2) Castaneda's stain (Bedson's modification) [25] This method is suitable for either egg-grown or mouse-grown agents Fix the smears in Weiss mordant solution A (formalin, 100 ml , glacial acetic acid, 7 5 ml). Wash thoroughly. Stain for at least 10 minutes in formol blue solution prepared as follows·

M/15 phosphate buffer, pH 7 0	90 ml
Unna's blue (or azure II, 1 per cent in methyl alcohol)	10 ml
Formalin	5 ml

Wash thoroughly Differentiate in 0 25 per cent aqueous safranine for 5 to 10 seconds

(3) Giemsa's stain This stain gives excellent permanent preparations, provided a reliable brand of stain (National Aniline and Chemical Company, Incorporated, New York) is used

(a) Giemsa's stock solution Dissolve 0 5 gm of Giemsa powder in 33 ml of highest purity glycerol at 55° to 60° C for 1½ to 2 hours To this add 33 ml of absolute methyl alcohol, acetone-free Mix thoroughly and allow to sediment overnight in a desiccator to prevent absorption of moisture Pour off into small bottles and stopper tightly

In preparing the stain for use, make up dilutions with absolutely neutral distilled water (orange with neutral red or purple with hematoxylin) or with buffered water A trace of lithium carbonate (1 per cent) will usually suffice to give the desired reaction to stock distilled water Buffered water solution is frequently preferred

(b) Buffered water solution according to Wilcox and Logan

1. Prepare M/15 NaH_2PO_4 (anhydrous) using 9 5 gm of the salt in 1 l of distilled water
2. Prepare M/15 NaH_2PO_4 and H_2O by dissolving 9 2 gm of the salt in 1 l of distilled water

To make buffered water with a pH of 7 2, mix 72 ml of (1) and 28 ml of (2) with 900 ml of distilled water

Although the rapid staining method ordinarily used for blood smears may yield satisfactory preparations, the slow method is recommended, since Giemsa's stain is largely used in studying the finer aggregates of viral colonies within the cells Following is a description of the slow method

Place the fixed thin films in a Coplin jar containing dilute Giemsa's staining solution (1 drop to 5 ml of neutral buffered water) Hold the jar in an incubator at 37° C overnight (20 hours) After removing the slides from the stain, rinse thoroughly with distilled water, then dry between blotting papers To differentiate the stains, the thin film is dipped rapidly in absolute alcohol (in case the preparation is overstained, use 95 per cent alcohol), it is then washed in distilled water, dried, and examined with an oil immersion lens Permanent preparations may be made by carefully removing the cedar oil Although unmounted dry specimens are more durable, the preparations may be mounted by applying a cover slip and using cedar oil or neutral balsam or Clarite (Neville Company, Pittsburgh) [38]

b Staining of sections The best solutions for staining sections are Giemsa, Heidenhain's classical iron hematoxylin, or Noble's stain, applied to material fixed in Zenker's, Bouin's, or Schaudinn's solutions Sections fixed in corrosive sublimate ($HgCl_2$) must be treated as follows

1 Remove paraffin from section with xylol, 5 minutes
2. Remove xylol with absolute alcohol and 95 per cent alcohol.
3 Transfer to 70 per cent alcohol tinged with Lugol's solution for 15 to 30 minutes, until light brown
4 Place in 95 per cent alcohol to remove iodine
5. Pass through 70 and 50 per cent alcohol to distilled water

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Unna's blue (or azure II, 1 per cent in methyl alcohol) .	10 ml
Formalin	5 ml

Wash thoroughly. Differentiate in 0 25 per cent aqueous safranine for 5 to 10 seconds

this infection. To the health officer, however, knowing the exact cause of certain forms of pneumonitis constitutes one of his responsibilities. The proper identification of the agent may be of real assistance to the epidemiologist in locating the source of infection and in learning more about the sources and circumstances under which infections are spread This provides a reasonable basis for preventive measures It is of great interest to the virologist concerned with the characteristics of the agent, knowledge of which may eventually lead to its control.

Isolation of the agent from the sputum of a person who does not have the clinical symptoms and signs of psittacosis suggests the existence of a latent infection or carrier stage. In this case repeated examinations of the sputum are indicated so that the patient who continues to excrete potentially infective agents may take the proper precautions

Isolating the agent from the spleen, liver, kidneys, or cloacal content of birds epidemiologically linked with a human infection is of definite value If the agent can be isolated from 10 to 20 per cent of a flock of pigeons or parakeets, it must be considered heavily infected and a potential source of infection for other birds and human beings

9 *Further identification of the isolated strains.* This is accomplished through the direct complement fixation test, using the isolated agent as an antigen, and through pathogenicity tests, tests of sensitivity to sulfonamides, cross-immunity tests, and toxin and infectivity neutralization tests

Mouse spleen or lung tissue or yolk sac material rich in elementary bodies can be converted into a cocto antigen according to procedures described (p 405) and that described by Davis,[39] and used with a high-titered psittacosis antiserum from hyperimmunized guinea pigs or naturally infected or hyperimmunized pigeons as a control Guinea pigs that have recovered from an infection with a psittacine or mammalian strain are injected with 2 to 3 ml of a 10 per cent suspension of virus intraperitoneally at weekly intervals, with periodic rests of 1 to 2 weeks Ten injections as a rule produce excellent sera [40]

a Pathogenicity tests Inoculation of mice, guinea pigs, parakeets, ricebirds, or pigeons is useful in distinguishing strains of avian from those of mammalian origin and to some extent those of psittacine from those of columban origin (Chart I). Appropriate dilutions and routes are used to make these distinctions

b. Sensitivity to sulfonamides A few strains of the group respond to the sulfonamides somewhat differently.[41,42] Until the sensitivity of a

6 Place in 0.5 per cent aqueous solution of sodium hyposulfite for 5 to 10 minutes.

7. Wash in running water

8 Transfer to distilled water, stain, differentiate, dehydrate, and mount according to the staining method chosen

(1) Polychromatic Giemsa stain (Wolbach's modification)

Beautiful sections are obtained after fixation of the tissues with corrosive sublimate Transfer the sections from distilled water to the following solutions:

Distilled water	100 ml.
Giemsa solution	60 drops
Methyl alcohol	10 ml
Aqueous sodium carbonate, 0 5 per cent	2 drops

Stain for 24 hours, renew the staining solution twice Rinse in distilled water and differentiate as follows

1 Immerse in 15 per cent colophonium in acetone for a few seconds

2 Transfer to the same reagent for a few seconds

3. Place in a mixture of acetone 70 parts and xylol 30 parts

4 Transfer to a mixture of equal parts of acetone and xylol.

5 Clear in xylol and mount in neutral cedar oil, never in balsam.

Control the progress of differentiation under the microscope.

A modification of Schaudinn's solution consisting of equal parts of saturated solution of mercury bichloride ($HgCl_2$) and acetone, with 5 per cent acetic acid, is particularly useful when using the Noble's stain described below.

(2) Noble's stain

Prepare sections from carefully embedded paraffin blocks and stain as follows

1. Steam section flooded with 1 per cent basic fuchsin solution over an alcohol lamp for 45 seconds

2. Decolorize and differentiate in 0.5 per cent citric acid solution for 1½ minutes

3 Wash in distilled water

4 Counterstain with 2 per cent aqueous orange G solution for 5 minutes

5 Rinse lightly and counterstain with 1 per cent aqueous methylene green solution for 5 minutes

6 Differentiate rapidly in 95 per cent alcohol

7. Pass rapidly through absolute alcohol into xylol

8 Mount in balsam or euparal

8. *Significance of the results.* This test provides the most certain basis for the diagnosis of psittacosis. To the patient, demonstration of the psittacosis agent in the blood or sputum is largely an academic matter, because the results are usually not available during the course of his acute illness, but that is true of all of the present means of diagnosing

characterize certain strains[43] A weakly virulent strain and its related types provoke immunity only against homologous challenge; a highly virulent strain may provoke immunity against all other members of the group. For example, mice resistant to the virulent 6BC strain resist challenge to large doses of a less virulent pigeon strain[44] The opossum strain, of low virulence, protects only against homologous challenge

Guinea pigs that survive infection with avian or mammalian strains resist severe challenge with the highly virulent Louisiana (Borg)[45,46] or egret (S E 45)[47] strain given intraperitoneally Immunization with the egret strain protects them against intraperitoneal challenge with the virulent turkey strain Their resistance to reinfection with avian strains has varied from 20 to 60 per cent.

These tests may prove useful in classifying members of the psittacosis group

d Toxin viral infectivity neutralization tests The toxin neutralization test with 9 different sera and 27 toxigenic strains[48] places these strains into six groups (1) the Louisiana pneumonitis agent (Borg), (2) the San Francisco agents, (3) the feline pneumonitis agent, (4) the meningopneumonitis virus of Francis and Magill, (5) 4 strains isolated from pigeons, and (6) 14 strains isolated from avian and human sources (some with unknown exposure) Some antisera are sharply specific for the homologous toxin, but the sixth group remains undifferentiated

A subsequent combination of the toxin neutralization with the viral infectivity neutralization technic has disclosed that the psittacosis group is made up of highly specific serotypes Neutralization tests to differentiate strains are feasible through use of specific rooster immune sera[49] The murine and feline pneumonitis agents are antigenically distinct Sera specific for these strains do not neutralize the 6BC, San Francisco, Illinois, or Nigg strains We have found the results more decisive when the neutralization was tested in mice, using the intravenous route The specific protective action of an antipsittacosis serum is more clearly defined by intravenous than by intranasal or intracranial injection[41] The protection is highly specific for most strains according to host origin. Antisera prepared with avian strains fail to protect against mammalian strains The strains of murine, feline, and ovine pneumonitis and of epizootic abortion of ewes are neutralized only by homologous antisera[50]

(1) Preparation of toxic suspensions.[48,51] The strains to be tested are prepared in infected yolk sacs of 6- to 7-day embryonated chicken eggs For harvesting yolk sacs, see p 407 In order to secure a potent

larger number of strains has been studied the differential value of this test cannot be ascertained.

c. Cross-immunity tests. Subcutaneous inoculation of mice, once or several times, with sublethal doses of live agents of this group or with large doses of killed agents, and subsequent challenge, may help to

CHART I

IDENTIFICATION OF PSITTACOSIS-LYMPHOGRANULOMA VIRAL AGENTS

	Pathogenicity-Virulence				Complement Fixation (Group Reaction)	Neutralization of	
	Mice Four Routes	Guinea Pigs Intraperitoneal	Pigeons Intracerebral	Ricebirds Parakeets Intramuscular		Toxin	Infection
Avian origin						*	
Psittacine	IP, IN, IC, SC* ±	0	±	+	+	Cross	Cross
Pigeon	IP ± IN, IC	0	+	+	+	Cross	Cross
Dove	IP ± IN, IC	0	+	+	+	Cross	Specific
Duck	IP ± IN, IC	0	±	+	+	Cross	Cross
Chicken	IN, IC	0	+	+	+	—	—
Turkey	IP, IN, IC, SC* ±	++++	0	+	+	Cross	Specific
Egret	IP, IN, IC, SC* ±	++++	±	+	+	Cross	Specific
Mammalian origin							
Mouse pneumonitis	IP, IN, IC,	0	0	0	+	—	Specific
Feline	IN	+++	—	0	+	Specific	Specific
Opossum	IP, IN, IC	+++	0	—	+	Specific	Specific
Calf, B E V.	IN	+++	—	0	+	Specific	Specific
Ewe; E.A E.	IN	+++	—	0	+	Cross	Cross
Human							
Borg, Louisiana	IP, IN, IC, SC	++++	±	+	+	Cross	Specific
S F.	IP, IN, IC, SC	±	±	+	+	Cross	Specific
Other strains	IP, IN, IC	±	± to +	+	+	Cross	Cross
Lymphogranuloma	IN, IC	±	0	0	Absorbed + Specific	Specific	Specific

0 = negative
— = not tested
* ± Carrier with occasional death.
+ Kills.
+++ 50 per cent or more deaths
++++ 100 per cent deaths.

2. *Skin sensitivity tests.* Sensitivity to presently available antigens occurs infrequently and is difficult to interpret. It always develops in patients with lymphogranuloma venereum, but the more acute nature of psittacosis and early treatment with effective antibiotics may be responsible for the irregularity or lack of allergy. This test is, of course, not suitable for early diagnosis It remains a subject for further investigation

III REFERENCES

1 Li

J A M A, 95 171–179, 1930

2 Binford, C H, and Hauser, G H An Epidemic of a Severe Pneumonitis in the Bayou Region of Louisiana III Pathological Observations Report of Autopsy on Two Cases with Brief Comparative Note on Psittacosis and Q Fever *Pub Health Rep*, 59 1363–1373, 1944

3 Meyer, K F Ornithosis and Psittacosis Chapter 22 in *Diseases of Poultry*, 3d ed edited by H E Biester and L H Schwarte Ames, Iowa The Iowa State College Press, 1952, pp 569–618

4 Meyer, K F, and Eddie, B Reservoirs of the Psittacosis Agent *Acta Trop*, 9 204–215, 1952

5 Baker, J A A Virus Causing Pneumonia in Cats and Producing Elementary Bodies *J Exper Med*, 79 159–172, 1944

6 McKercher, D G Feline Pneumonitis I Immunization Studies in Kittens *Am J Vet Res*, 13 557–561, 1952

7 Gonnert, R Die Bronchopneumonie, eine neue Viruskrankheit der Maus *Zentralbl f Bakt, Abt 1, Orig*, 147 161–174, 1941 Gonnert, R Uber ein neues, dem Erreger des Lymphogranuloma inguinale ahnliches Mausevirus *Klin Wchnschr*, 20 76–78, 1941. Gonnert R Uber einige Eigenschaften des Bronchopneumonie virus der Maus *Zentralbl f Bakt, Abt 1, Orig*, 148 331–337, 1942

8 Nigg, C, and Eaton, M D Isolation from Normal Mice of a Pneumotropic Virus Which Forms Elementary Bodies *J Exper Med*, 79 497–510, 1944

9 Karr, H V A Study of Latent Pneumotropic Virus of Mice *J Infect Dis*, 72 108–116, 1943

10 York, C J, and Baker, J A A New Member of the Psittacosis Lymphogranuloma Group of Viruses That Causes Infection in Calves *J Exper Med*, 93 587–604, 1951

11

Disease *J Am Vet M A*, 122 294–299, 1953

12 Roca García, M Viruses of the Lymphogranuloma Psittacosis Group Isolated from Opossums in Colombia Opossum Virus A *J Infect Dis*, 85 275–289, 1949

toxin, eggs must be watched carefully, and the yolk sacs must be harvested when the embryos are moribund or just dead. Yolk material is carefully cleared from membranes. Smears are prepared to check for viral elements, and cultures are made to detect bacterial contaminants. Yolk *sacs are then ground thoroughly, made up in 20 per cent suspension* in beef heart broth, and held in the refrigerator for 2 to 8 days.

(2) Diluent. A 0.1 M phosphate buffer at pH 6.8 containing 0.2 per cent gelatin is the diluent of choice.

$NaH_2PO_4 \cdot H_2O$	6.0 gm.
Na_2HPO_4 (anhydrous)	7.95 gm
Bacto gelatin	2.0 gm.
Distilled water	1 l.

tilled water is added to make 1 liter. The solution is dispensed in suitable working amounts and sterilized in the autoclave at 15 pounds for 15 minutes.

E. Other Diagnostic Tests

The hemagglutination inhibition test and the conglutinating complement absorption test have been used, and group specificity has invariably prevailed. Antigenic differences have been few, minor, and not constant enough to be of practical value.[33,52] Hemagglutinin is laborious to prepare, is unstable on storage, and is infectious. Many sera have proved anticomplementary in the conglutinating complement absorption test These tests are no more useful in the serodiagnosis of psittacosis than the direct complement fixation test.

1. *Micro-agglutination test.* The specific antibody reaction in the agglutination test, studied to only a limited degree until recently, is the subject of renewed attention.[53] Formalin-killed, purified, and concentrated suspensions of elementary bodies prepared from infected mouse lungs,[54] from allantoic fluid or even yolk sac material, are specifically agglutinated by sera from infected chickens, turkeys, mammals, or man. Production of uniformly sensitive specific antigen for the micro-agglutination test by the methods used in rickettsial infections continues to offer many technical problems. Although significant results are being reported,[55] the agglutination test is not yet available for routine laboratory use.

32 Ka
firmation of the Specificity and Epidemiologic Application of the Test. J Infect Dis 87 24–36 1950

33 R

34

35 He
M Sc, 23 107–110, 1945

36

37.

38

39

40 Bedson, S P
Path, 14 162–170, 1933

41 Wiseman, R W, Meiklejohn, G, Lackman, D B, Wagner, J C, and Beveridge, G W Studies on the Chemotherapy of Viruses in the Psittacosis-Lymphogranuloma Group II Effect of Penicillin and Sulfadiazine on Seven Strains in Mice *J Immunol*, 54 9–16, 1946

42 Meyer, K F, and Eddie, B Human Pneumonitis Viruses and Their Classification *Arch ges Virusforsch*, 4 579–590, 1952

43 Wagner, J C, Golub, O J, and Andrew, V W *Cross-Immunity Studies Between* Virus Strains in the Psittacosis-Lymphogranuloma Venereum Group. *J Infect Dis*, 84 41–46, 1949

44 Smadel, J. E, Wall, M J, and Gregg, A An Outbreak of Psittacosis in Pigeons, Involving the Production of Inclusion Bodies and Transfer of the Disease to Man *J Exper Med*, 78 189–204, 1943

45 Olson, B J, and Larson, C L An Epidemic of a Severe Pneumonitis in the Bayou Region of Louisiana. IV A Preliminary Note on Etiology. *Pub Health Rep*, 59 1373–1374, 1944

46 Olson, B J, and Larson, C L An Epidemic of a Severe Pneumonitis in the Bayou Region of Louisiana V Etiology *Pub Health Rep*, 60 1488–1503, 1945

13. Stamp, J. T., McEwen, A. D., Watt, J. A. A., and Nisbet, D. I. Enzootic Abortion in Ewes. I. Transmission of the Disease. *Vet. Rec.*, 62 251–254, 1950. McEwen, A. D., Stamp, J. T., and Littlejohn, A. I. Enzootic Abortion in Ewes. II. Immunization and Infection Experiments. *Vet Rec.*, 63 197–201, 1951. McEwen, A. D., Littlejohn, A. I., and Foggie, A. Enzootic Abortion in Ewes. Some Aspects of Infection and Resistance. *Vet Rec.*, 63 489–492, 1951. Barwell, C. F., and Bishop, L. W. J. Virus of Enzootic Abortion in Ewes. Antigenic Relationship with Viruses of the Psittacosis Group. *Nature*, 167,998, 1951. Monsur, K. A., and Barwell, C. F. Observations on the Antigenic Relationship Between the Virus of Enzootic Abortion in Ewes and Viruses of the Psittacosis-Lymphogranuloma Group. *Brit. J. Exper. Path.*, 32 414–421, 1951.
14. McKercher, D. G. A Virus Possibly Related to the Psittacosis-Lymphogranuloma-Pneumonitis Group Causing a Pneumonia in Sheep. *Science*, 115:543–544, 1952.
15. Meyer, K. F. Psittacosis Group. *Ann New York Acad Sc.*, 56 545–556, 1953.
16. Bedson, S. P., and Western, G. T. Observations on Virus of Psittacosis. *Brit. J. Exper. Path.*, 11 502–511, 1930.
17. Barwell, C. F. Antigenic Components of Psittacosis Virus. *Nature*, 162:460–461, 1948.
18. Barwell, C. F. Some Observations on the Antigenic Structure of Psittacosis and Lymphogranuloma Venereum Viruses. II. Treatment of Virus Suspensions by Various Reagents and the Specific Activity of Acid Extracts. *Brit. J. Exper. Path.*, 33 268–279, 1952.
19. Bedson, S. P., Western, G. T., and Levy Simpson, S. Further Observations on the Aetiology of Psittacosis. *Lancet*, 1 345–346, 1930.
20. Bedson, S. P., Western, G. T., and Levy Simpson, S. Observations on the Aetiology of Psittacosis. *Lancet*, 1 235–236, 1930.
21. Bedson, S. P. Use of the Complement-Fixation Reaction in the Diagnosis of Human Psittacosis. *Lancet*, 2 1277–1280, 1935. Bedson, S. P. Observations on the Complement-Fixation Test in Psittacosis. *Lancet*, 2 1477–1480, 1937.
22. Meyer, K. F., and Eddie, B. The Value of the Complement Fixation Test in the Diagnosis of Psittacosis. *J. Infect. Dis.*, 65 225–233, 1939.
23. Meyer, K. F., Eddie, B., and Yanamura, H. Complement Fixation Test with Tissue-Culture-Antigens as Aid in Recognizing Latent Avian Psittacosis (Ornithosis). *Proc. Soc. Exper. Biol. and Med.*, 41 173–176, 1939.
24. Smadel, J. E., Wertman, K., and Reagan, R. L. Yolk Sac Complement Fixation Antigen for Use in Psittacosis-Lymphogranuloma Venereum Group of Diseases. *Proc. Soc. Exper. Biol. and Med.*, 54 70–74, 1943.
24a. Miles, J. A. R. Observations on Complement Fixation and Complement Fixation Inhibition Using Certain Avian Sera. *Australian J. Exper. Biol. and M. Sc.*, 32 57–68, 1954.
25. [illegible]
26. [illegible]
27. [illegible] tion, 1948, pp 1–45
28. Nigg, C., Hilleman, M. R., and Bowser, B. M. Studies on Lymphogranuloma Venereum Complement-Fixing Antigens. I. Enhancement by Phenol or Boiling. *J. Immunol.*, 53.259–268, 1946.
29. Whitney, E., and Gnesh, G. M. Potent Psittacosis Antigens Free of Anticomplementary Activity. *Proc. Soc. Exper. Biol. and Med.*, 87 356–360, 1954.
30. Bedson, S. P. Personal communication to the authors, 1953.
31. Sigel, M. M., and Pollikoff, R. Reduction of Group Reactivity of Complement-Fixing Antigen of Meningopneumonitis Virus by Potassium Periodate. *Proc. Soc. Exper. Biol. and Med.*, 84 517–520, 1953.

EPIDEMIOLOGY OF PSITTACOSIS AND ORNITHOSIS

HOUSE EPIDEMICS, OCCUPATIONAL AND ACCIDENTAL INFECTION

Microbacterium multiforme (Filterable)

PSITTACIDAE

COLUMBIDAE

Incubation time - 7-31 days

High temperature with slight remission

Toxemia entirely out of proportion to physical findings

Non productive pneumonia or pneumonitis

Sputum — Droplet or contact

Blood virulent 1st–3rd day

Sputum virulent 5th – 23rd day

Regularly in fatal cases

Autopsy, virus in lung, spleen and liver

Complement fixation of serum — positive 8th–15th day

INFECTED DROPPINGS
DUST AEROGENIC ROUTE

Size of birds in relation to incidence and importance

Experimentally infected Mice, Guinea Pigs, Hamsters, Pocket Gophers, Rabbits, Monkeys

FIGURE 1

47 ...dson, M E. Isolation of a ... ets. *Proc Soc. Exper. Biol.*

48 ...sittacosis-Lymphogranuloma ... e Toxin Neutralization Test *J Infect Dis*, 86 241–250, 1950

49 Hilleman, M R., and Gordon, F. B Immunologic Relations of the Psittacosis-Lymphogranuloma Group of Viral Agents. *Proc. Soc. Exper Biol. and Med.*, 56 159–161, 1944 Hilleman, M. R. Immunological Studies on the Psittacosis-Lymphogranuloma Group of Viral Agents. *J. Infect Dis*, 76.96–114, 1945. St John, E., and Gordon, F. B. Studies on the Immunological Relationships of the Psittacosis-Lymphogranuloma Venereum Group of Viruses. *J. Infect Dis*, 80. 297–306, 1947

50 M

51 Ra ... 323–338, 1942

52 Stoker, M G P, Coombs, R. R. A., and Bedson, S. P. The Application of the Conglutinating Complement Absorption Test to Virus Systems. *Brit. J Exper Path*, 31 217–232, 1950

53

54 Fulton, F and Begg, A M The Antigenic Structure of Typhus Rickettsiae in Chemotherapeutic and Other Studies of Typhus Chapter 5 in *M. Res Coun Spec. Rep Ser*, No 255, 1946, pp. 163–191 Giroud, P., and Jadin, J. Comparaison entre différents tests pour le diagnostic de la fièvre Q, réactions allergiques, fixation du complément et agglutination des rickettsies. *Compt rend Acad sc*, 230 2347–2348 1950 Giroud, P, and Jadin, J Importance des micro-agglutinations pour le diagnostic sérologique des infections provoquées par des éléments virulents à la limite des rickettsies *Compt rend Soc biol*, 148 1157–1158, 1954

55 Babudieri, B, Bussinello, E, Bajocchi, E, and Lusiani, G. B. Ricerche epidemiologiche e sierologiche su di alcuni casi di ornitosi in provincia di Modena. *Settimana Med*, 42 463–466, 1954 Babudieri, B. Immunologie der Rickettsiosen. *Ztschr Immunitätsforsch*, 112 182–201, 1955 Giroud, P, Roger, F., and Dartois, N. Comportement des sérums de divers animaux domestiques vis-à-vis de l'antigène de la psittacose. *Compt rend Acad sc*, 238 419–420, 1954.

STEPS TO DETERMINE THE PRESENCE OF PSITTACOSIS VIRAL AGENTS INFECTED BIRDS (OR MAMMALS)

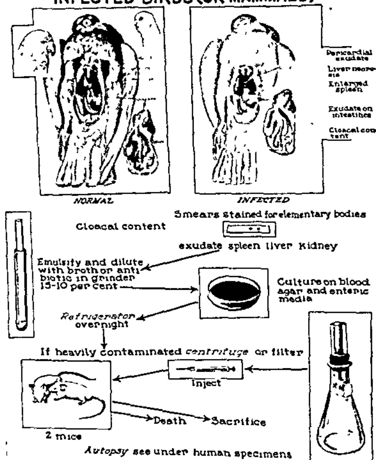


FIGURE 3

STEPS TO DETERMINE THE PRESENCE OF PSITTACOSIS VIRAL AGENT HUMAN SPECIMENS

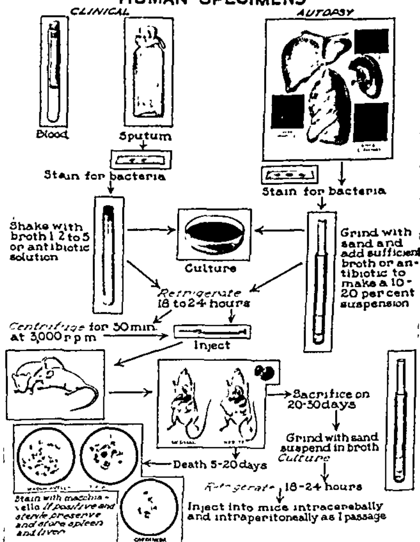


FIGURE 2

STEPS TO DETERMINE THE PRESENCE OF PSITTACOSIS VIRAL AGENTS INFECTED BIRDS (OR MAMMALS)

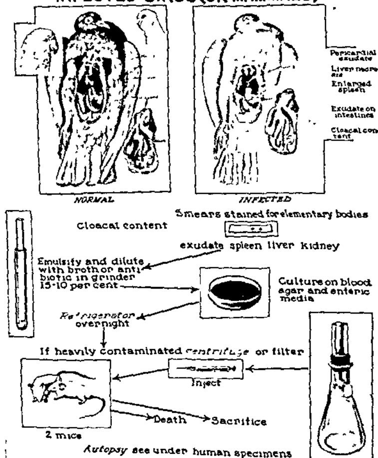


FIGURE 3

TRACHOMA

PHILLIPS THYGESON, M.D

Clinical Professor of Ophthalmology, University of California
School of Medicine, San Francisco, California

I INTRODUCTION
- A General Statement
- B Clinical Features and Diagnosis
- C The Virus
- D Transmission

II ISOLATION AND IDENTIFICATION OF VIRUS
- A Precautions for Workers
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- C Preparation and Examination of Epithelial Scrapings
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- D Preparation and Examination of Follicular Expressions
  - 1 Preparation
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- E Preparation and Examination of Secretion Smears and Cultures
- F Serologic Procedures
- G Animal Inoculations for Experimental Purposes
  - 1 Source of material
  - 2 Selection of animals for inoculation
  - 3 Routes of inoculation
  - 4 Experimental trachoma

III REFERENCES

I. INTRODUCTION

A. General Statement

TRACHOMA is a virus disease of world-wide distribution which attacks the conjunctiva and cornea specifically, with serious cicatricial sequelae leading to diminished vision or even blindness. The disease may start either acutely or insidiously, but if untreated always runs a chronic course over a period of many years. Cases are known to have persisted during a lifetime. When the disease affects young children, however, there is a tendency to spontaneous healing, which has been estimated to be as high as 30 per cent in some series.

B. Clinical Features and Diagnosis

The most characteristic feature of the early acute phase of trachoma is a papillary hypertrophy of the conjunctiva, accompanied by an abundant conjunctival exudate in which neutrophilic leukocytes are predominant. In its chronic phase follicular hypertrophy is the most characteristic feature. The cornea is involved simultaneously with the conjunctiva and eventually shows subepithelial infiltrates, pannus, and frequently corneal ulceration. Cell necrosis with cicatrization is highly characteristic and useful in diagnosis. In severe cases conjunctival cicatrization may result in deformity of the lids, corneal cicatrization is the cause of the diminished vision or blindness. A diagnostic clinical feature is the predominant involvement of the upper parts of the conjunctiva and cornea; in inclusion conjunctivitis, which is due to a virus of the same group, the maximum localization of the virus is in the conjunctiva of the lower lid.

In most instances trachoma can be diagnosed on the basis of these clinical features alone, but laboratory procedures are distinctly valuable in difficult or complicated cases. Of greatest diagnostic importance clinically are (1) the follicular hypertrophy involving the upper tarsal conjunctiva, (2) the development of pannus, and (3) the conjunctival and corneal cicatrization. With the aid of the slit-lamp and corneal microscope, these characteristic changes can be recognized very early in the disease.

Secondary bacterial infection is very common in trachoma. In countries such as Egypt it may complicate the disease in 100 per cent of cases, the most important bacteria being *Neisseria gonorrhoeae* and the bacilli of the Hemophilus group. In this country the most common secondary invader is *Staphylococcus aureus*, with *Diplococcus pneumoniae*, *Hemophilus influenzae*, *Hemophilus conjunctivitidis* (Koch-Weeks bacillus), and *Moraxella lacunatus* (diplobacillus of Morax-Axenfeld) next in order of frequency.

Trachoma is favorably influenced by treatment with the sulfonamides, of which sulfanilamide and sulfadiazine are most commonly employed, and with the broad spectrum antibiotics. Recent studies suggest that erythromycin and magnamycin are also therapeutically active.

C. The Virus

The specific cause of trachoma is a virus of the psittacosis-lymphogranuloma venereum group of viruses The taxonomy of this group of agents was long in dispute, but in 1945 Moshkovskii [1] suggested the family name of Chlamydozoaceae and the generic names of *Chlamydozoon* and *Miyagawanella* The agent of trachoma he named *Chlamydozoon trachomatis* and the agent of inclusion blennorrhea, a closely related disease, he named *Chlamydozoon oculogenitale*. The virus of trachoma is believed to attack only conjunctival and corneal epithelium, and it is assumed that all subepithelial changes result from the presence of a diffusible toxin [1a] liberated by the virus in the epithelium. It has been impossible to demonstrate the virus morphologically in subepithelial tissues or to infect the conjunctiva of the experimental animal except by way of the epithelium

The virus appears typically in the form of elementary bodies morphologically identical with those of the other members of the psittacosis-lymphogranuloma venereum (Chlamydozoaceae) group Just as also occurs in the course of the intracellular development of the other viruses of this group, large swollen forms appear, which are known as initial bodies and which have somewhat different tinctorial properties The intracellular colonies of the virus, or inclusion bodies, are of diagnostic value in the human disease and need only be distinguished from the virus colonies of inclusion blennorrhea The inclusion consists of the elementary and initial bodies embedded in a matrix made up principally of glycogen. When stained with Giemsa's stain, or other similar dye, the young inclusions, consisting principally of initial bodies, tend to be more basophilic than the larger inclusions, consisting principally of elementary bodies It is the elementary body which is the filtrable form, but filtrations are obtained with difficulty.[2]

There are now a number of claims for the cultivation of trachoma virus But their validity has not yet been established, and it can be said that no one has yet been able to maintain a strain in serial culture There has been no difficulty, however, in growing its normal host cells, the conjunctival and corneal epithelium.[3]

D. Transmission

The infectivity of trachoma is known to be very low in the chronic form of the disease but may be high in the acute form. Transmission is

from eye to eye and requires close contact. The virus is quite sensitive to drying, and transfer tends to occur only under very unhygienic conditions The familial character of the disease is well demonstrated in such countries as North Africa and even among the Indians of our southwest. In these areas the father in the family may have the disease and the children remain free, but if the mother is infected the children are almost invariably also infected. Trachoma may spread simultaneously with bacterial conjunctivitis, particularly gonorrheal ophthalmia and Koch-Weeks conjunctivitis Recent studies in Egypt[4] appear to give conclusive evidence that trachoma, along with the acute ophthalmias (gonococcic conjunctivitis, Koch-Weeks conjunctivitis), may be transmitted by flies. Experimental fly control measures in isolated villages have been remarkably successful in reducing the incidence of all these types of conjunctivitis It is known that wrestlers are frequently infected, probably as a result of the close contact in wrestling and the common practice among wrestlers of massaging their opponents' eyeballs violently to induce a vagus reflex.

II ISOLATION AND IDENTIFICATION OF THE VIRUS

A Precautions for Workers

Unlike psittacosis virus, trachoma virus is not dangerous to work with, for its communicability is low, and ordinary hand precautions suffice to protect the worker. Accidental transmission of the disease to doctors and nurses has been known to occur but only during surgical procedures such as curettage and expression in which infected material has struck the eye The wearing of glasses and the avoidance of contact of the hands with the eyes should provide sufficient protection for all practical purposes

B Types of Procedure

Laboratory procedures in trachoma fall into two categories according to their purpose, as follows.

1. *For diagnostic purposes.*

a. Epithelial scrapings for the morphologic demonstration of the virus and the recognition of such complicating factors as allergy and tear deficiency.

b. Follicular expressions for the detection of specific cytologic changes.

c. Secretion smears and cultures for the determination of secondary bacterial infection.

2 *For experimental purposes.*

a Animal inoculation (monkeys and apes).

As previously noted, the diagnosis of trachoma is primarily clinical, only early and atypical cases requiring laboratory procedures Examination of epithelial scrapings is first in order of diagnostic importance, and examination of expressed follicular contents, second Only in extremely acute cases can the free virus be seen in secretion smears, but both smears and cultures are useful in the diagnosis of secondary bacterial infection Knowledge of the presence or absence of secondary infection, and of its nature, if present, is a prerequisite to the application of adequate therapy

Biopsy material is of little value in trachoma, although the virus can be seen in properly stained sections from the conjunctiva. For methods of staining sections the reader is referred to the chapters on "Psittacosis" and "Lymphogranuloma Venereum" since the tinctorial properties of trachoma virus resemble very closely those of the viruses of these two diseases

The inoculation of experimental animals has no diagnostic utility in trachoma The technics are discussed in this chapter solely for the benefit of the experimental worker

No serologic procedures of diagnostic importance have been developed as yet

C PREPARATION AND EXAMINATION OF EPITHELIAL SCRAPINGS

1 *Sources of material* The virus, which is to be found in both the conjunctiva and cornea, is most abundant in the early acute stages of the disease, and there is general agreement that much more of it is to be found in the conjunctiva of the upper tarsus and fornix than of the lower tarsus and fornix Epithelial scrapings from the upper tarsus and upper fornix, therefore, constitute the best source of virus material and the most important preparations from trachoma They are much better than biopsy material or follicular expressions since the virus appears to be limited strictly to the epithelium and is more abundant in the superficial layers than in the deep. Scrapings are best taken with a platinum spatula from the previously anesthetized conjunctiva and from the area of greatest disease activity. This is usually the tarsal conjunctiva, and more particularly its upper border, but scrapings from the upper fornix are also valuable.

In the diagnosis of secondary bacterial infection, examination of scrapings from the lid margins is indicated when there is a complicating blepharitis

2. Staining methods. The scrapings should be placed on clean, grease-free, dust-free slides. They are best fixed for 5 minutes with absolute methyl alcohol, acetone-free, and stained with Giemsa's stain. Wright's stain may be used as a substitute, but it is more difficult to obtain debris-free slides with this stain. Since the tinctorial properties of trachoma virus are almost identical with those of the other members of the psittacosis-lymphogranuloma venereum group of viruses, other stains such as the Castaneda, the Macchiavello, and the Victoria blue can be used, but they offer no real advantage and have in general been less satisfactory than Giemsa's stain For the technic of staining with the Castaneda and Macchiavello stains, the reader is referred to the chapters on "Psittacosis" and "Lymphogranuloma Venereum."

Giemsa's stain gives excellent, permanent preparations provided a reliable stain is employed and care taken in having a neutral distilled water as diluent. A stock preparation of Giemsa's solution can be obtained from various sources,* or the stock-staining solution can be prepared from powder.† One-half gm. of the powder is dissolved in 33 ml. of the highest purity glycerol at 55° to 60° for 1½ to 2 hours. To this is added 33 ml. of absolute methyl alcohol, acetone-free. The solution is then mixed thoroughly and allowed to sediment overnight in a desiccator to prevent absorption of moisture It is then poured off into small bottles and stoppered tightly.

Dilutions of stock Giemsa's stain are made with neutral distilled water or buffered water. If the water is allowed to become acid, the stain will shift to the red side, and if allowed to become alkaline, will shift to the blue. If there is any difficulty in obtaining satisfactory distilled water, a buffered neutral water can be used. Distilled water is best kept in pyrex bottles, since ordinary glass bottles have a tendency to liberate alkali into the water The preparation of buffered water is described in the chapter on "Psittacosis."

The fixed epithelial scrapings are stained as follows:

a. Place the slides in a Coplin jar in a dilute Giemsa's solution (1 drop to 2 ml. of neutral distilled water)

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* Gradwohl Laboratories, St Louis

† National Aniline and Chemical Co, Inc, New York City

b Place the jar in an incubator at 37° C. for at least one hour.

c. Remove stain debris by rinsing the slide rapidly in two changes of 95 per cent alcohol.

d Dry and examine.

During the staining process care must be exercised to prevent dust particles from settling on the slide. If permanent preparations are desired, place a drop of cedar oil on the slide and cover with a thin cover slip If more rapid staining is required, fix with May Grunwald solution for 3 minutes and stain with concentrated Giemsa's solution (2 drops to 1 ml of neutral distilled water) for 15 minutes

Wet fixation has been advocated by Lindner[5] for special studies of the inclusion bodies, but it is not a diagnostic procedure. The iodine stain described by Rice,[6,7] which brings out the carbohydrate matrix of the inclusion body, has some usefulness, however, since it enables one to scan a slide rapidly under low power, the reddish brown glycogen color of the matrix being easily recognizable under low magnification An inclusion body stained with one of the other dyes can more easily be missed unless oil immersion is used It should be pointed out, however, that many of the small inclusion bodies do not have carbohydrate matrices and could, therefore, also be missed when stained with iodine The number of inclusion bodies in any particular case seems to be in proportion to the clinical severity of the trachoma As many as 20 per cent of the conjunctival epithelial cells in the pure, acute, exudative type may be affected whereas in chronic trachoma of very low intensity it may be necessary to search many slides before a typical inclusion body can be found In such cases it is clearly helpful to scan the slides under low power magnification prior to the use of the oil-immersion lens

3 *Morphology of the virus particles* In epithelial scrapings from the affected conjunctiva or cornea, the virus appears in its more characteristic form, the elementary body, a minute coccus-like body about 0 2 to 0 25 μ in diameter, either singly, in clusters, or in clumps The virus also appears in the form of initial bodies, which are larger, more bluish, bipolar-staining bodies, with coccobacillary morphology, and as intracellular masses of either elementary or initial bodies or both The elementary bodies tend to stain purple with Giemsa's stain and the initial bodies, pure blue Electron microscopy of the elementary and initial bodies has not added significant information as to their internal structure

While the virus can be seen extracellularly in the acute stages of the disease, in the chronic stages it can usually be identified only in the

form of the intracellular inclusion bodies. It is the inclusions, therefore, that have the greatest diagnostic value. As with other members of the psittacosis-lymphogranuloma venereum group of viruses, trachoma virus undergoes a definite sequence of morphologic change within affected cells. This sequence is believed to run as follows: An elementary virus particle is phagocytosed by an epithelial cell; the particle then swells to form a coccobacillary body many times its original size. This is the initial body, which then divides to form more bodies of comparable size. As the divisions continue, the cytoplasm of the cell is replaced with the inclusion mass, the elements of division become smaller and smaller, and finally the elementary body stage is reached again. From observations made during the incubation period of experimental trachoma in man, this sequence of morphologic change from elementary body through initial body and back to elementary body, with rupture of the cell and liberation of the virus particles, requires about 48 hours.

As previously pointed out, the inclusion mass is embedded in a carbohydrate matrix, which takes a characteristic glycogen stain with Lugol's solution. Finding of the typical cytoplasmic inclusion body limits the diagnosis to trachoma or inclusion blennorrhea. Differentiation between these two diseases, on laboratory evidence, must be based on cytologic differences rather than on any difference in the morphology of the inclusion bodies. The cytologic differences are best observed in follicular expressions, as will be discussed in Section B below.

4. *Morphology of the inclusion body* Unfortunately, the trachoma inclusion body, which has such great diagnostic value, is not the only cytoplasmic element found in conjunctival scrapings Differentiation must be made from a number of pseudoinclusions. Among these is extruded nuclear material. This is usually easy to identify since the extruded material retains the tinctorial properties and characteristic texture of the original nucleus. Other pseudoinclusions are phagocytosed nuclear material, pigment from heavily pigmented individuals such as the Indian or Negro, and stain debris. This last frequently simulates initial body inclusions, although the experienced observer can always make the differentiation. In view of these sources of possible confusion, however, the laboratory diagnosis of trachoma should, for all practical purposes, depend on the finding of the characteristic elementary body inclusion. This inclusion, with its typical elementary bodies (Fig 1) and carbohydrate matrix (Fig. 2), cannot be confused with any of the so-called pseudoinclusions

As previously noted, the number of inclusions in pure trachoma varies directly with the amount of exudation. Active, secreting trachoma, excluding those cases in which secondary infection is the cause of the exudation, always shows abundant inclusion bodies. Follicular trachoma without much secretion, on the other hand, may have few, if any, inclusions. Certain old, cicatricial cases of low activity have been reactivated by the topical use of cortisone, with the development of abundant inclusions. The use of this cortisone effect as a diagnostic test and a test of activity has been suggested.[8,9]

It is of interest that in trachoma with inclusion bodies the percentage of cells infected is always greater in scrapings from the upper tarsal conjunctiva than in scrapings from the lower conjunctiva, and that this

5. *Recognition of complicating factors.* Epithelial scrapings are also useful in the determination of such complicating factors as allergy and tear deficiency. A high eosinophil count always indicates an allergy and most commonly a vernal catarrh. The finding of keratinized epithelium in conjunctival scrapings is of importance as an indicator of tear deficiency, common in old cicatricial trachoma, and should always be checked by the Schirmer test, by which the output of tears in a measured period of time can be determined. Tear deficiency makes the eradication of secondary bacterial infection much more difficult.

D. Preparation and Examination of Follicular Expressions

1. *Preparation.* As noted above, expressed trachoma follicle material shows specific cytologic changes which are of especial value in the differential diagnosis of trachoma and inclusion blennorrhea.[10,10a] Preparations are best obtained by means of the Prince ring forceps or an ordinary chalazion curette. With one or the other of these instruments the material is gently expressed from a follicle on the anesthetized conjunctiva, placed on a clean slide, and stained with Giemsa's stain by the method described for staining epithelial scrapings.

2. *Nature of the follicle.* Diagnostically significant is the fact that the trachoma follicle, due to the characteristic necrotic changes which occur within it, is typically soft and gelatinous in contrast to the hard follicles of all types of follicular conjunctivitis and folliculosis. The trachoma follicle is, therefore, very easily expressed with the ring forceps,

and the expressed material, when suitably stained with Giemsa's stain, shows a very characteristic cytologic picture. In marked contrast to the easy expressibility of the soft trachoma follicle, the nontrachomatous follicle, such as that of inclusion conjunctivitis, is hard and difficult to express. In many instances the whole follicle must be literally torn from the conjunctiva. This probably explains why follicular material from inclusion conjunctivitis presents a cytologic picture quite different from that of trachoma.

3. *Cytology of the expressed material.* Examination of expressed follicular material from trachoma reveals the following picture: (*a*) numerous macrophages, their cytoplasm loaded with broken-down nuclear material, (*b*) numerous pale-staining, seminecrotic, large mononuclear germinal center cells, probably lymphoblasts, which differ sharply in appearance from the similar cells found in expressed material from *nontrachomatous follicular conjunctivitis*, (*c*) *an extraordinary amount* of cell debris scattered throughout the slide, and (*d*) a few scattered plasma cells and lymphocytes, typically far less numerous than the large mononuclear cells.

In expressed follicular material from inclusion conjunctivitis, on the other hand, the following changes are noted: (*a*) macrophages, when present, are very few in number and tend to be much smaller than the giant macrophages of trachoma; (*b*) there are no necrotic changes in the large mononuclear germinal center cells and little, if any, cellular debris, and (*c*) small round cells, mostly lymphocytes, predominate and constitute the most striking cytologic feature.

E. Preparation and Examination of Secretion Smears and Cultures

The cytology of the secretion in trachoma is significant only in the fact that the neutrophilic leukocytes are predominant, and that this finding, although also characteristic of inclusion conjunctivitis, differs sharply from the findings in follicular conjunctivitis caused by typical viruses such as epidemic keratoconjunctivitis virus and herpes simplex virus, both of which produce predominantly a mononuclear cell reaction.

The primary purpose of examining secretion smears and cultures in trachoma, however, is to determine the presence or absence of secondary bacterial infection or allergy There is indeed such an exceedingly high incidence of secondary infection in trachoma that smears and cultures should be made and examined routinely in order to ensure the application of adequate therapeutic measures This is particularly

important in old cicatricial cases in which secondary infection may keep the trachoma active In such cases topical application of a sulfonamide or antibiotic may be very much more effective than their systemic administration

Secretion smears are best fixed by heat and stained by Gram's method When an acute bacterial infection is superimposed on a trachoma, the organisms most commonly seen will be the pneumococcus, bacilli of the Hemophilus group, and *Staph aureus* In rare instances *Strep viridans* or *Strep hemolyticus* may be found

The occurrence of a conjunctival eosinophilia is indicative of a complicating conjunctival allergy, the most common type being vernal catarrh

Routine cultures are best taken on blood agar plates by means of the wet swab technic (cotton applicators moistened with 1 per cent glucose broth) It is useful to divide the plate into four parts, two quadrants being used for each eye, one of the two for material from the conjunctiva, the other for material from the lid margins Anaerobic cultures are usually not indicated Special culture media are employed only in cases of suspected secondary infection with the gonococcus or the diphtheria bacillus, both of which are extremely uncommon complications in this country

F. Serologic Procedures

So far no serologic procedures of diagnostic significance in trachoma have been developed Serologic findings have indeed been almost totally lacking,[11] due no doubt to the fact that trachoma virus is strictly epitheliotropic It has so far been impossible to demonstrate viricidal antibodies in the blood serum, and second attacks of the disease occur without modification of symptoms No hypersensitivity skin test, such as the Frei test for lymphogranuloma venereum, has ever been demonstrated Rake, Shaffer, and Thygeson[12] found that serum from trachoma cases gave low complement fixation titers for lymphogranuloma venereum antigen and this has been confirmed recently by Kornblueth *et al*[12a] and Bietti *et al*[12b] No trachoma antigen studies have been reported, but this is not surprising in view of the fact that trachoma virus cannot yet be cultivated as a routine laboratory procedure.

G Animal Inoculation for Experimental Purposes

1 *Source of material* In animal experiments, epithelial scrapings taken from the area of greatest disease activity are best transferred directly without preliminary dilution Since the virus is never very abundant in scrapings, dilution tends to reduce its infectivity, but if it is necessary to transport the material or to make delayed inoculations, a concentrated suspension of epithelial scrapings in saline will usually remain active for periods up to 24 hours, particularly if kept on ice. At room temperature the virus is rapidly inactivated[13] but it may survive indefinitely in the frozen state[11]

2 *Selection of animals for inoculation.* There has been considerable dispute as to which animals can be infected. It is now generally agreed, however, that only monkeys and apes contract experimental trachoma, and that the experimental disease is unlike the human disease in that severe complications such as cicatrization and pannus never occur. In fact, experimental trachoma in monkeys is strictly a follicular conjunctivitis which runs a spontaneous course to healing, and bestows no immunity. In no experimental animal is it possible, therefore, to make an unequivocal clinical diagnosis of trachoma. Furthermore, as has been repeatedly pointed out by such observers as Julianelle,[11] Wilson,[14] and Bland,[15] extreme care must be used in interpreting the results of monkey inoculations with trachomatous materials because of the animals' susceptibility to a spontaneous follicular disease which may simulate experimental trachoma. This disease, known as spontaneous folliculosis, has vitiated many experiments in trachoma and has made human inoculations necessary for final determinations. Closely resembling folliculosis in humans, the spontaneous disease consists of a follicular hypertrophy of the conjunctiva, particularly of the fornices, with minimal inflammatory signs

The chimpanzee is by all odds the best experimental animal for trachoma, but the prohibitive cost of its purchase and care necessitates the use of monkeys or baboons for most research. The baboon (*Papio hamadryas*) has been more satisfactory than the *Macacus rhesus* since it is less susceptible to spontaneous folliculosis and develops an experimental disease easier to recognize clinically Any animal selected for inoculation should first be examined carefully for evidence of the spontaneous disease

3 *Routes of inoculation* Direct surface inoculation of the conjunctiva is more satisfactory than subconjunctival injection. The material containing the virus can be rubbed lightly over the conjunctiva Light massage definitely facilitates infection, although positive inoculations have been obtained as a result of simple instillation of the infectious material into the conjunctival sac

4. *Experimental trachoma.* The incubation period of experimental trachoma in the rhesus monkey and baboon is usually from 1 to 3 weeks. The onset is always insidious, acute trachoma never occurring in the experimental animal, which should be observed for a full month after inoculation before the experiment is declared negative. As noted above,

experimental trachoma is always self-limited, but its duration varies greatly from several months in the rhesus monkey to a year in the baboon. It is extremely difficult to demonstrate the virus morphologically in epithelial scrapings from experimental trachoma, but even when it is present in too small amounts to be seen, transmission is still possible[16] Animals which have recovered from experimental trachoma are not immune to the disease, and a second infection appears to run very much the same clinical course as the first.

III. REFERENCES

1 Moshkovskii, Sh D Tsitotropnie vozbyditeli infektsii i mesto rikkettsii v sisteme snlamjdozoa *Usp Sovrem Biol*, *19* 1–44, 1945

1a Mitsui, Y, Tanaka C, Yamashita, K and Hanabusa, J Toxin Like Substance of Trachoma Virus *Arch Ophth*, 52 72–76, 1954

2 Thygeson, P, Proctor, F I, and Richards, P Etiologic Significance of the Elementary Body in Trachoma *Am J Ophth*, 18 811–813, 1935

3 Thygeson, P Cultivation of Trachomatous Conjunctival Epithelium *in Vitro* *Arch Ophth*, 21 229–234, 1939.

4 Lyons, F Maxwell The Double Problem of Acute Conjunctivitis and Trachoma in Egypt, Epidemiology and Recent Experiments in Prevention *Rev Internat Trachome*, 30 341–351 (No 3) 1953

5 Lindner, K Die freie Initialform der Prowazekschen Einschlusse *Arch f Ophth*, 76 559–567, 1910

6 Rice, C E Carbohydrate Matrix of Epithelial-Cell Inclusion in Trachoma *Am J Ophth*, 19 1–8, 1936

7 Thygeson, P Matrix of Epithelial Cell Inclusion Body of Trachoma *Am J Path*, 14 455–462, 1938

8 Freyche, M J, Nataf, R, Maurin, J, and Delon P Clinical and Laboratory Research in Connexion with a Criterion of Cure in Trachoma *W H O /Trachoma/*35, Feb 9, 1953

9 Thygeson P Criteria of Cure in Trachoma with Special Reference to Provocative Tests *Rev Internat Trachome*, 30 450–464 (No 4) 1953

9a Braley, A Intracellular Bodies of the Conjunctival Epithelial Cells *Arch Ophth*, 24 681–690 1940

10 Thygeson, P The Cytology of Conjunctival Exudates *Am J Ophth*, 29,1499–1512 1946

10a Thygeson, P The Cytologic Diagnosis of Trachoma *Rev internat trachome* In press

11 Julianelle, L A *The Etiology of Trachoma* New York, The Commonwealth Fund, 1938 263 pp

12 Rake, G., Shaffer, M F, and Thygeson, P Relationship of Agents of Trachoma and Inclusion Conjunctivitis to Those of Lymphogranuloma Psittacosis Group *Proc Exper Biol and Med*, 49 545–547, 1942

12a Kornblueth W, Feigenbaum, A., and Bernkopf, H The Presence of Complement-fixing Antibodies to the Group-specific Antigen of Psittacosis–Lymphogranuloma Venereum Group in the Sera of Trachomatous Patients *Trans XVII Intern. Congress of Ophth*, 1954

12b Bietti, G B Babudieri B and Pannarale, M R Further Contributions on the Subject of the Existence of Antigenic Affinity between the Agents of Trachoma and Those of Venereal Lymphogranuloma Psittacosis To be published.

13 Stewart, F H Experimental Pathology of Trachoma *Mem. Ophthalmic Lab., Giza, Ann Rep* (Appendix 1, 1937), 12 1–27, 1938
14 Wilson, R. P Pathology of Trachoma. *Tr Internat. Ophth. Cong.*, 1937 (Le Trachome) 5 88–100, 1938 Also *Bull Ophth Soc Egypt*, 29:1, 1936.
15 Bland, J O W Aetiology of Trachoma Critical Review of Present Knowledge. *Mem Ophthalmic Lab., Giza, Rep* (1939–1944), 14 89–106, 1945
16 Bland, J O W Spontaneous Folliculosis of Conjunctiva in Grivet and Vervet Monkeys (*Lasiopyga griseoviridis syn Cercopithecus aethiops* and *L. pygerythra syn. C. pygerythra*) and Susceptibility of Grivet to Trachoma Virus. *Mem. Ophthalmic Lab., Giza Rep* (1939–1944), 14 72–86, 1945



Fig 1 Epithelial cell with elementary body inclusion Giemsa stain



Fig 2 Same cell showing carbohydrate matrix Iodine stain

INCLUSION BLENNORRHEA

PHILLIPS THYGESON, M D.

Clinical Professor of Ophthalmology, University of California School of Medicine, San Francisco

I INTRODUCTION

A GENERAL STATEMENT

INCLUSION BLENNORRHEA,[1] or inclusion conjunctivitis, which is also known under a variety of names such as paratrachoma, genital trachoma, acute follicular conjunctivitis (inclusion body type), and swimming-pool conjunctivitis, is a virus disease whose principal manifestations are seen in the newborn baby. It also occurs in the adolescent or adult, however, sometimes as a form of swimming-pool conjunctivitis, sometimes as an accidental self-infection from the genitourinary disease,

and again without any known source of infection. The disease is self-limited, running a course from acute onset through a chronic phase to healing in from 3 to 9 or 10 months. A few cases are on record in which the clinical manifestations persisted for more than a year. The sulfonamides and the medium- and broad-spectrum antibiotics are effective in therapy

B. Clinical Features and Diagnosis

In the newborn infant, inclusion blennorrhea appears from the 5th to the 11th day after birth as an acute papillary conjunctivitis characterized by abundant purulent secretion The acute phase, which usually lasts from 10 days to 2 weeks, sometimes has alarming inflammatory manifestations, such as pseudomembrane formation When these subside there is a chronic inflammatory phase which gradually resolves over a period of several months There are no corneal complications and no sequelae except in those cases which have had marked pseudomembrane formation Some of these show fine conjunctival scars.

In the adult the clinical manifestations of the disease differ so sharply from those in the newborn baby that at one time they were thought to constitute a different disease entity Inclusion conjunctivitis in the adult[2,3] is usually an acute follicular conjunctivitis without the abundant exudate so characteristic of the infection in the newborn Occasionally, however, a more severe form is seen in which papillary hypertrophy is predominant In this type the exudate is more abundant, although never in the amount seen in the inclusion blennorrhea of the newborn

Unlike trachoma, which it resembles in many respects, inclusion blennorrhea cannot be diagnosed with certainty on clinical grounds alone. Laboratory procedures are essential, the diagnosis being based on the finding of cytoplasmic inclusion bodies identical with those of trachoma In the inclusion blennorrhea of the newborn, the finding of these inclusions is pathognomonic since in this country trachoma rarely occurs in the newborn baby and then never as an acute conjunctivitis It is the acute inclusion conjunctivitis of the adult that must be differentiated from trachoma The clinical findings then complete the picture since the virus of inclusion conjunctivitis does not produce corneal changes, and trachoma virus invariably does Laboratory differentiation based on the cytologic picture of expressed follicular material is also possible, as was described in the section on trachoma

C The Virus

The specific cause of inclusion blennorrhea is a virus of the psittacosis-lymphogranuloma group of viruses, now known as *Chlamydozoon oculogenitale*[4] and id[illegible]
intracellular colonies [illegible]
the inclusion bodies of [illegible]
cyc[illegible]
as [illegible]
Like the [illegible]

other viruses of the group, the inclusion blennorrhea inclusion has a glycogen-containing matrix. In general, inclusion blennorrhea virus is much more abundant in the lesions, particularly in the newborn baby, than is trachoma virus in the lesions of trachoma. This relative abundance of virus probably accounts for the ease with which filtrations[2,4,8] have been accomplished in inclusion blennorrhea as opposed to the difficulty encountered with them in trachoma

The pathologic lesions produced by inclusion blennorrhea virus are very similar to those produced by trachoma virus except that the necrotic changes in the cells of the subepithelial tissues which occur in trachoma, probably as the result of a soluble toxin liberated by the virus in the epithelium, are never produced by inclusion blennorrhea virus

All attempts to cultivate inclusion conjunctivitis virus have failed up to the present time Unlike the other viruses of the group, neither trachoma virus nor inclusion conjunctivitis virus has been cultivable in the yolk sac of the developing chick embryo

So far no one has reported the preservation of inclusion conjunctivitis virus over a long period of time, although it should be possible to accomplish with such modern methods as desiccation *in vacuo* It is certain that inclusion conjunctivitis virus, like trachoma virus, is highly susceptible to drying Its viability when moist, on the other hand, is clearly indicated by its transmissibility[9] in the water of swimming pools At room temperature, however, it fails to remain active, even in the moist state, for periods longer than several hours Only at refrigerator temperatures will suspensions of the virus remain active for periods up to 24 hours

D Transmission

Although *Chlamydozoon oculogenitale* is pre-eminently a cause of ocular infection, its main habitat is the genitourinary tract[10] In the male it causes a benign urethritis, which heals spontaneously after a period of several months, and in the female it causes a type of cervicitis limited to a narrow strip of epithelium at the external os and to all practical purposes asymptomatic

Eye infections occur almost invariably as a result of transfer from the genitourinary tract to the eye Eye-to-eye transmission[11] has been accomplished experimentally a number of times but has seldom occurred spontaneously. In the newborn the infection is transferred from the cervix of the mother to the baby's eye during birth; in the adult it results from a transfer of genitourinary material to the eye, either directly

by way of the fingers or indirectly through water as in the case of swimming-pool infections The epidemiology is strikingly similar to that of gonorrheal conjunctivitis. In fact the only difference is the swimming-pool transmission, which seems to be based on the fact that although both agents are highly susceptible to drying, inclusion conjunctivitis virus is considerably more viable when wet than is the gonococcus. The virus does not seem to be affected by penicillin when used as a substitute for the classical Credé silver nitrate prophylaxis.

Recent studies by Ormsby *et al.*[12] indicate that inclusion conjunctivitis in the subsiding stage can be reactivated by the topical use of cortisone.

II. ISOLATION AND IDENTIFICATION OF VIRUS

A. Precautions for Workers

Workers with inclusion blennorrhea virus need only exercise ordinary caution to avoid accidental infection When scrapings or expressions are being taken from patients or experimental animals, the wearing of glasses should prevent the spattering of infectious material into the eyes. This is particularly important when working with the newborn baby since dammed-up secretion very often spurts suddenly from the eyes when the lids are opened The worker should avoid rubbing his eyes with his fingers while working with the disease, but ordinary washing of the hands with soap and water is sufficient to remove the virus The disease can be said to be in a minor way an occupational disease of obstetricians and gynecologists. Accidental infection of mothers and nurses has also occurred.

B. Types of Procedure

The following laboratory procedures are useful for diagnostic and experimental purposes in inclusion blennorrhea:

1. *Epithelial scrapings* for morphologic demonstration of the virus.

2. *Follicular expressions* for cytologic studies and differential diagnosis from trachoma.

3. *Secretion smears* for diagnosis in acute cases and in inclusion urethritis in the male.

4. *Animal inoculations* for experimental purposes and in the diagnosis of inclusion urethritis in the male.

As with trachoma virus, the strictly epithelial localization of inclu-

sion blennorrhea virus makes epithelial scrapings much more effective for its morphologic demonstration than follicular expressions. The latter are useful, however, for cytologic studies and for differentiating inclusion conjunctivitis from trachoma on the basis of cell reaction. For a description of the method of preparing follicular expressions and of the cytology of the expressed material from both inclusion blennorrhea and trachoma, the reader is referred to the section on trachoma.

Secondary bacterial infection is uncommon in inclusion conjunctivitis and of no significance in its management, although before the Credé method of prophylaxis came into general use cases of mixed inclusion blennorrhea and gonorrheal ophthalmia were occasionally seen Routine examination of secretion smears and cultures are, therefore, of only secondary importance. In acute cases smears may suffice for diagnostic purposes, but epithelial scrapings are always to be preferred Smears have special utility in the diagnosis of inclusion urethritis, however, since urethral scrapings are usually impossible to obtain

Animal inoculations are made principally for experimental purposes but have some practical utility in the diagnosis of the genitourinary disease, especially the nonspecific urethritis in the male in which microscopic diagnosis is difficult

C Preparation and Examination of Epithelial Scrapings

1 *Sources of material* The best source of virus is pooled epithelial scrapings from cases of inclusion blennorhea in the newborn Although this is the best source of a high concentration of virus, less highly concentrated virus-containing material can also be obtained from inclusion conjunctivitis of the adult, more abundantly from the papillary type, and from the genitourinary disease in both male and female As it is usually not possible to obtain urethral scrapings in the male, the urethral exudate must be examined instead The virus is often in such low concentration in the exudate that it is quite difficult to see it in smears, although the same material will usually be infectious for baboons Cervical infections, on the other hand, yield epithelial scrapings which frequently show a high concentration of virus

As was mentioned in the section on trachoma, the two viruses differ with respect to their maximum localization, trachoma virus tending to localize in the conjunctiva of the upper lid predominantly and inclusion blennorrhea virus in the conjunctiva of the lower lid predominantly. Inclusion blennorrhea scrapings should, therefore, always be taken from

by way of the fingers or indirectly through water as in the case of swimming-pool infections The epidemiology is strikingly similar to that of gonorrheal conjunctivitis. In fact the only difference is the swimming-pool transmission, which seems to be based on the fact that although both agents are highly susceptible to drying, inclusion conjunctivitis virus is considerably more viable when wet than is the gonococcus The virus does not seem to be affected by penicillin when used as a substitute for the classical Crédé silver nitrate prophylaxis.

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4. *Animal inoculations* for experimental purposes and in the diagnosis of inclusion urethritis in the male

As with trachoma virus, the strictly epithelial localization of inclu-

of inclusion blennorrhea consists at least in large part of glycogen, which takes a reddish brown stain with Lugol's solution

D Animal Inoculations

1 *Experimental inclusion conjunctivitis* Like trachoma virus, inclusion conjunctivitis virus has been transmitted only to monkeys and apes Unlike experimental trachoma, however, experimental inclusion conjunctivitis (Fig 4) resembles closely the human disease, particularly the acute follicular conjunctivitis of the adult Thus, inoculation of the conjunctiva of the baboon with infectious material results, after an incubation period of a week to 10 days, in a follicular conjunctivitis, rather subacute in character, which runs a course over a period of several months and heals spontaneously without residuals During the first few weeks of the experimental disease, the virus is readily demonstrable in epithelial scrapings As the disease progresses virus demonstration in scrapings becomes increasingly difficult, but the existence of the infection can be established by transfer of the disease to other experimental animals Like experimental trachoma, experimental inclusion conjunctivitis heals spontaneously without immunity Baboons, for example, have been infected with the virus repeatedly, and the disease has run a characteristic course with each succeeding inoculation without significant modification

Animal inoculation appears to have no practical significance except in the diagnosis of the genitourinary disease, particularly of the nonspecific urethritis in the male Owing to the small amount of virus in the exudate, as well as to the difficulty of obtaining epithelial scrapings from the urethra, baboon inoculation has been found to be a practical procedure

2 *Sources of confusion* As in the study of trachoma by animal inoculation, the main source of confusion is the presence of a spontaneous folliculosis which may simulate experimental trachoma or experimental inclusion conjunctivitis. Fortunately, the skilled observer finds the similarities not too great Experimental inclusion conjunctivitis, with its subacute onset and characteristic course, combined with the presence of inclusion bodies in epithelial scrapings in the early stages of the disease, is rather readily differentiable from the spontaneous folliculosis. Every effort should be made, however, to obtain animals which are free from the spontaneous disease

the conjunctiva of the lower lid and fornix and during the period of greatest disease activity; that is, the early acute stage, which usually lasts from 1 to 2 weeks The amount of virus diminishes rapidly as the disease progresses.

As noted above, inclusion blennorrhea virus in normal salt solution can be kept refrigerated for periods up to 24 hours. Under ordinary circumstances, however, material should be used immediately after its collection.

2. *Staining methods* Inclusion blennorrhea virus has the same tinctorial properties as the other viruses of the psittacosis-lymphogranuloma venereum group. While Giemsa's method, described in detail in the section on trachoma, has been used more widely in the study of this virus than any other method, Wright's stain has also been employed with success, and the reader is referred to the chapters on "Psittacosis" and "Lymphogranuloma Venereum" for descriptions of this and a number of other elementary body stains which have been used in the study of various members of the group

The iodine stain, which brings out the carbohydrate matrix of the inclusion body, has less utility in this disease than in trachoma, since the inclusions are usually sufficiently numerous to be picked up readily in Giemsa-stained preparations under high magnification The glycogen staining of these two types of inclusion bodies, however, is interesting as the chief property differentiating them from the inclusions of other viruses of this group.

3 *Morphology of the virus* Inclusion conjunctivitis virus is indistinguishable morphologically from psittacosis virus, lymphogranuloma venereum virus, and the other viruses of the group. In Giemsa-stained smears of exudate from acute cases it appears in the form of elementary and initial bodies, a combination which gives a picture so characteristic as to be diagnostic In Giemsa-stained epithelial scrapings the virus is seen extracellularly as elementary bodies (Fig. 2) and intracellularly as

[illegible]

cent of the epithelial cells may be infected. Multiple infection of epithelial cells also occurs, sometimes as many as 3 or 4 inclusions developing in a single cell Like the trachoma virus inclusion, but unlike the inclusions of the other viruses of the group, the matrix of the inclusion body

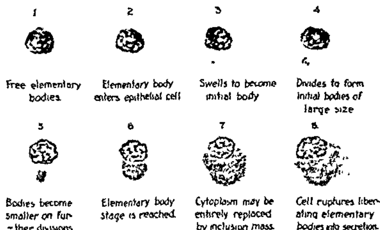


Fig 1 Development cycle of virus of inclusion conjunctivitis Schematic drawing

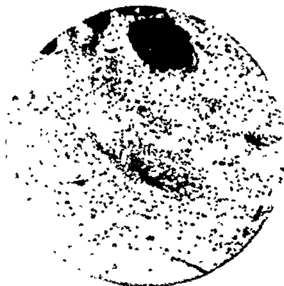


Fig 2 Elementary bodies in scrapings from cervix in inclusion virus cervicitis of mother of infant with inclusion blennorrhea. Giemsa stain

In inoculation experiments on the baboon the only virus diseases to be ruled out are trachoma and lymphogranuloma venereum. Unfortunately, there is at present no known way to distinguish experimental trachoma from experimental inclusion conjunctivitis except by human inoculation, since both diseases in animals are essentially an acute follicular conjunctivitis without pannus or scarring. Lymphogranuloma venereum virus might cause confusion, since it is occasionally found in nonspecific urethritis. Experimental lymphogranuloma venereum conjunctivitis of the baboon, however, bears little or no clinical resemblance to the acute follicular conjunctivitis of inclusion blennorrhea in baboons. Another way of distinguishing these 2 viruses would be by means of the differential staining of the inclusion bodies. The inclusion body of lymphogranuloma venereum does not have the carbohydrate, glycogen-staining matrix characteristic of the inclusion bodies of inclusion conjunctivitis and trachoma.

E. Serologic Procedures

Inclusion conjunctivitis virus has no more ability to induce immunologic reactions than has trachoma virus. There is no hypersensitivity skin reaction and, except for the presence of group complement-fixing bodies as demonstrated by Rake, Shaffer, and Thygeson,[13] there have been no serologic findings. These complement-fixing bodies are of low order and have no role in the laboratory diagnosis of the disease.

III. REFERENCES

1. Lindner, K. Gonoblennorrhöe, Einschlussblennorrhöe und Trachom. *Arch. f. Ophth.*, 78:345–380.
2. Thygeson, P. Etiology of Inclusion Blennorrhea. *Am. J. Ophth.*, 17:1019–1035, 1934.
3. Thygeson, P., and Mengert, W. F. Virus of Inclusion Conjunctivitis: Further Observations. *Arch. Ophth.*, 15:377–410, correction 734, 1936.
4. Moshkovskii, Sh. D. Tsitotropnie vozbyditeli infektsii i mesto rikkettsii v sisteme [illegible]
5. [illegible]
6. [illegible] 690, 1912.
7. Tilden, E. B., and Gifford, S. R. Filtration Experiments with Virus of Inclusion Blennorrhea. *Arch. Ophth.*, 16:51–54, 1936.
8. Julianelle, L. A., Harrison, R. W., and Lange, A. C. Studies on Inclusion Blennorrhea, Experimental Considerations of Etiology. *Am. J. Ophth.*, 21:1230–1241, 1938.
9. Thygeson, P., and Stone, W., Jr. Epidemiology of Inclusion Conjunctivitis. *Arch. Ophth.*, 27:91–122, 1942.
10. Lindner, K. Zur Aetiologie der gonokokkenfreien Urethritis. *Wien. klin. Wchnschr.*, [illegible]
11. [illegible] *phth.*, 27:833–846, 1944.
12. [illegible] G. G., Lloyd, L. A., and Hassard, J. [illegible] *Am. J. Ophth.*, 35:1811–1814 (Dec.) 1952.
13. Rake, G., Shaffer, M. F., and Thygeson, P. Relationship of Agents of Trachoma and Inclusion Conjunctivitis to Those of Lymphogranuloma-Psittacosis Group. *Proc. Soc. Exper. Biol. and Med.*, 49:545–547, 1942.

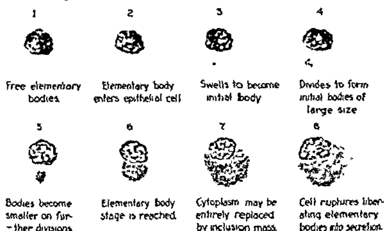


FIG 1 Development cycle of virus of inclusion conjunctivitis Schematic drawing

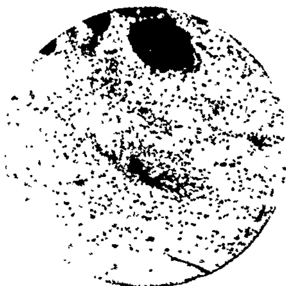


FIG 2 Elementary bodies in scrapings from cervix in inclusion virus cervicitis of mother of infant with inclusion blennorrhea Giemsa stain



Fig 3 Inclusion bodies produced by inclusion conjunctivitis virus Drawn from a magnification of 1250 diameters

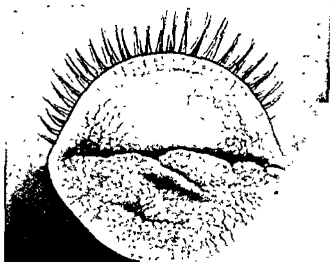


Fig 4 Experimental inclusion conjunctivitis of the baboon

LYMPHOGRANULOMA VENEREUM

GEOFFREY RAKE, M.B., B.S.

Research Professor of Microbiology in Medicine, School of Medicine, and Research Professor of Microbiology in Medicine, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

I. INTRODUCTION

LYMPHOGRANULOMA VENEREUM, *lymphogranuloma inguinale*, or climatic bubo is a venereal disease. In its characteristic form it begins with a primary lesion—a small and transient vesicle on the genitalia—which, especially in the female, is frequently unnoticed. This is followed in from 6 to 50 days by painful swelling of the regional inguinal lymph nodes, which will usually suppurate and drain through the overlying skin if left untreated. The whole course is commonly run without fever, but an acute febrile attack may be associated with the primary lesion, or a low grade and prolonged fever may complicate the adenitis.

A CLINICAL FEATURES

More rarely the disease is protean. There may be acute septicemic episodes at the onset, particularly in laboratory infections where dosage may be an important factor, and these may result in meningitis or pneumonitis The primary attack may occur in the mouth, due to unnatural sexual practices or laboratory infections, and is sometimes highly destructive in this site, or it may occur in the eye also as a result of unnatural practices, where it produces a highly destructive disease distinguishable with difficulty from acute trachoma. Particularly in the female the spread of the disease to the pelvic lymph nodes, or a primary implantation in the rectum, leads to chronic elephantiasis of the genitalia, esthiomene, anal fistula, and rectal stricture.

The responsible agent *Miyagawanella lymphogranulomatosis* [1] can be isolated from a primary lesion in the eye and probably from other primary lesions if these are seen and recognized. It is more usually obtained, however, from the secondarily infected tissue—lymph nodes, anorectal tissue, or spinal fluid. It should be looked for in any case of

[illegible]

the anus or rectum. The exclusion of other venereal diseases producing similar lesions is important The disease though more frequent in Negro males is not limited to any race or to sex.

B. THE AGENT

The usual form of the agent is a small coccoid body about 300 mμ in diameter, staining with aniline dyes, gram negative, and visible with the light microscope. It can also be studied with the electron microscope

where it appears spherical, with limiting membrane and central irregular denser area. Under the conditions required for electron microscopy, the elementary body appears wrinkled and collapsed as though a loss of fluid had occurred. Inside a cell this elementary body may not be distinguishable as such since, particularly in the early stages of infection, it is enclosed within an inclusion body which consists of a dense matrix derived either from the agent or from the cell—possibly from both. Particularly during the early stages of the infection, initial bodies about 1 μ in diameter are often found. Each initial body appears to consist of an elementary body with a surrounding mantle of matricial material.

C. Pathologic Lesions

In the early stages of infection the characteristic lesion is that in the inguinal nodes. They become enlarged and matted together with a dense periadenitis. At a later stage, abscess formation is usual, and complicating hemorrhage may occur. On section various stages of nonspecific inflammation are found, even to complete destruction, but the typical lesions are small foci of epithelioid cells and some giant cells, not unlike tubercles or early gummata. These epithelioid foci are the site of multiplication of the agent and in them originate the abscesses which complicate the later picture of the lesion. Within the epithelioid monocytes may be found the characteristic inclusions within which, as has been indicated above, the elementary bodies are embedded.

II. ISOLATION AND IDENTIFICATION OF THE AGENT

A. Precautions for Workers

The agent is highly infectious for man. Laboratory infections have occurred through lesions on the hands and through the oral cavity.[2] Vaccination with such vaccines as have been available has not proved an efficacious prophylactic. The newer antibiotics such as tetracyclin, however, are highly effective in controlling the clinical disease. It is questionable how frequently such chemotherapy actually clears the tissues of the agent, and serologic studies suggest that a permanent or very prolonged carrier state usually occurs. In handling material suspected of containing this agent, particularly in high concentration, it is recommended that the workers wear goggles or translucent face masks. Rubber gloves may be preferred but are likely to confer a false sense of security and to render the worker clumsier. The agent is considerably more resistant and stable than is indicated in the literature.

B. Collection and Preparation of Material

As stated above, the most common sources of material for isolation of the agent are bubo pus, biopsy material from inflamed node or anorectal tissue, and cerebrospinal fluid. When collecting any such tissues one should exert great care to avoid bacterial contamination. When such precautions are adopted spinal fluid should always be free of bacteria; tissue or pus from nodes free in 80 to 85 per cent of instances [3,4] and anorectal tissue usually contaminated. To ensure the highest proportion of positive isolations, however, aseptic technic, rather than indiscriminate use of sulfonamides or antiseptics, should be used. The specimens should be shipped in closely sealed glass containers within double mailing cases. Freezing the material is desirable but should be avoided unless maintenance of the frozen state can be assured until the specimen reaches the laboratory.

If samples are potentially contaminated with bacteria, further treatment is required before they can be used for inoculation. The agent of lymphogranuloma venereum is susceptible to commercial penicillin, but the concentrations required before any effect is observed (in vitro 2,250 u/ml,[5] and in vivo 50 u/ml.[6]) are much higher than those required for antibacterial action. Also streptomycin in our experience has had no effect on the agent even with concentrations as high as 3,500 u/ml in vitro or 20,000 u/ml. in vivo.[6] Material in which bacterial contamination is suspected may, therefore, be treated with final concentrations as high as 1,000 units of penicillin and 3,000 units of streptomycin per ml. in vitro before animal inoculation

C. Inoculation and Observation

Two inoculation technics have proved most effective in isolation of the agent—the intracerebral inoculation of mice and the injection into the yolk sac of chick embryos. Although the last-named method is more sensitive for the detection of small numbers of infective units of established strains, the greater susceptibility of the chicken embryo, compared with that of mice, to bacterial contaminants is a great disadvantage. Moreover, other work has suggested that the intracerebral inoculation of mice is more sensitive in the isolation of new strains than is the yolk sac technic.[4] For both these reasons mice would seem to be the animals of choice in the inoculation procedure. Guinea pigs have also been used[7] but seem to offer no advantages over mice

Most mice are susceptible to the agent inoculated intracerebrally,

but the Swiss albino is markedly so and is suggested for use Care must be taken to avoid stocks of mice spontaneously infected with *Miyagawanella bronchopneumoniae*[8] (the agent of mouse bronchopneumonia—Gonnert[9]) or any other member of the genus *Miyagawanella*[8] related to the agent of lymphogranuloma venereum

Of mice Young 14 to 16 gm animals are preferred The sample, if of pus or tissue treated as suggested with penicillin and streptomycin, is prepared in both 10 and 50 per cent suspensions in normal egg yolk by grinding with alundum Spinal fluid is used undiluted and diluted 1 10 Quarter ml syringes with ½-inch 27-gauge needles are used The mice are lightly anesthetized, the fur over the skull moistened with 70 per cent alcohol on a swab, and each of the animals then inoculated with 0 04 ml, with the needle inserted barely through the skull At least five mice are inoculated with each dilution They are then observed carefully at least twice a day for the next 10 days Specifically infected mice will show on the 2d, 3d, or subsequent days ruffled fur, loss of weight, and a hunched-back gait Such mice should be killed, and the brains removed aseptically Smears or, better, impressions of pieces of the cerebral cortex and meninges are made on a clean slide and stained with Macchiavello or Giemsa stain (see p 458) The rest of the brain is ground sterilely without abrasive, and a 10 per cent suspension is prepared in broth or normal yolk *This material is passed to 14 to 16 gm animals as above and into eggs* by the yolk sac route If no sickness develops, and this is particularly liable to occur in the first or isolation passage, the brains of the mice are nevertheless passed on the 10th day to new mice and to eggs by the yolk sac route

The number of "blind" passages to be made is open to question In our experience, however, if a strain of the agent of lymphogranuloma venereum is not established by the third passage, no strain has ultimately been derived no matter how many passages are made

2 Of chick embryos For primary or subsequent inoculation into the yolk sac of chick embryos, eggs of 6 days' prior incubation are chosen They are candled to ensure live, healthy embryos, and the air sac is marked off A drill hole over the end of the air sac is made up to but not through the shell membrane Material prepared as for intracerebral inoculation into mice is used, and 1 ml of each dilution is inoculated into the yolk sac of each of 5 eggs The eggs are candled twice a day Deaths within the first 48 hours are almost certainly nonspecific or are due to bacterial contamination, but all eggs are harvested, smears are prepared from the yolk sacs and stained, and the yolk sacs are kept until a microscopic diagnosis is made All yolk sacs from embryos dying after 96 hours, if not bacterially infected, are passed in 10 per cent suspension in normal yolk to 3 normal embryos by the yolk sac route In our experience 2 further passages have established all strains which will become established.

D Pathologic Specimens and Smears

1. Preparation Smears or impressions of mouse cerebrum or embryo yolk sac are prepared on clean glass slides, they are then fixed with light heat by passing the slide rapidly several times through a Bunsen

flame. *Before making a smear of yolk sac, excess yolk should be removed* by light impression on a gauze square Several stains are suitable, particularly Giemsa and Macchiavello. In general the Macchiavello stain has proved the more satisfactory and is used routinely.

2. Stains.

a *Giemsa stain for smears.* The smears are immersed in 95 per cent ethyl alcohol The slide is then flooded with Giemsa stain* diluted with equal parts 1.20,000 Na_2CO_3 solution Warm slide to gentle steaming and allow to cool for 10 minutes Wash vigorously with hot running tap water to remove precipitate. Blot dry.

b *Macchiavello stain for smears.*

(1) 0.25 per cent solution of basic fuchsin in phosphate buffer M/5 pH 7 4 Grind fuchsin in mortar and add buffer gradually

(2) 0 5 per cent citric acid solution

(3) 1 per cent aqueous methylene blue

The heated and cooled smears are flooded with solution 1 filtered through filter paper (Schleicher and Schull No 595) directly onto the slide. After 4 minutes the stain is washed off with the citric acid Wash rapidly in water, stain for 10 seconds in methylene blue, wash in water and dry

Smears should be examined with a good *oil immersion lens* at a magnification not less than 900. The elementary bodies, *ca.* 300 mμ in diameter, and the less numerous initial bodies, *ca.* 1 μ in diameter, will be seen, some within cells but for the most part lying free. With Macchiavello stain the elementary bodies are red (a few will be blue), and the initial bodies blue or red. Within the cells the former often appear as dense clusters within vacuoles or embedded, and scarcely visible, within the matrix of inclusion bodies. When free and not very numerous, as is often the case with impressions of mouse brains, they will tend to occur in loosely scattered groups in one out of many fields When more numerous, as from heavily infected yolk sacs, they are seen in pairs, chains, or densely packed morulae as well as singly.

Fixed specimens of brain or yolk sac may also be prepared for microscopic examination, *but these are usually not so informative* as are smears, and, furthermore, entail delay. Noble's stain[10] is suggested as the most satisfactory for stained preparations, and, for this, formalin or, preferably, Helly's fluid fixation is required, with subsequent paraffin embedding.

c. *Noble'*
solution and
1½ minutes

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* Giemsa's spirochete stain from Hynson, Westcott, and Dunning, Baltimore, Maryland, has given satisfactory results

5 minutes Rinse lightly and counterstain with 1 per cent methyl green for 5 minutes Differentiate rapidly in 95 per cent alcohol, pass rapidly through absolute alcohol into xylene, and mount in balsam

bodies are stained red with Noble's stain and may appear in dense clusters within vacuoles or may be embedded in the capsule-like matrix of the plaques, which stains green For more detailed description of the microscopic appearance, see Rake and Jones [11] Cell granules and cellular debris may cause trouble in diagnosis for the less experienced investigator, but the characteristic size, distribution, and staining of the bodies in heavily or moderately infected tissues is unlikely to be confused with anything else, particularly by those with previous experience Pappenheimer, Molloy, and Rose have described the appearance in the yolk cells of granules produced by certain technics of fixation and staining, the nature of which is uncertain [12] These might cause confusion where encountered but normally do not do so

Characteristic though the morphology and the staining may be, it is unfortunately shared by all the *Miyagawanellae* and even by the *Chlamydia* such as *C trachomatis* or *C oculogenitale* (agent of inclusion blennorrhea[8]). In order, therefore, that identification may be complete the agent must be isolated Even if the mouse is used for primary isolation, adaptation to the yolk sac of the chick embryo should be carried out early Yolk sac material is easier to handle and contains nearly a million times as much of the agent per gram as does mouse brain

In those cases in which successful isolation is not achieved, positive diagnosis of the case from which the tissue or other material came may be [illegible]

granuloma [illegible] the unknown sample contains inactivated agent or soluble antigen,[3] cases of lymphogranuloma will react to it in the same manner as to the antigens usually employed for skin test (see p 461)

E. Identification

There are two principal ways in which specific identification can be obtained after an agent is isolated.

1. *Toxin-antitoxin.* The first is the toxin-antitoxin neutralization test.[13,14] *Miyagawanellae lymphogranulomatosis*, in common with the

other *Miyagawanellae* which have been studied, produces a specific toxic factor.[13,14] The lethal effect of this on intravenous inoculation can be neutralized specifically by homologous antitoxin produced in rabbits or in chickens. Neutralization can be carried out in vivo by passive transfer of protection or in vitro. For practical purposes in vitro is preferable.

Equal amounts of given dilutions of antitoxic serum in normal yolk and embryo fluids and of toxin diluted in the same yolk and fluids to contain 4 M.L.D. per 0.5 ml are thoroughly mixed and allowed to stand for 2 hours at room temperature. Twelve to 14 gm mice are inoculated intravenously with 0.5 ml amounts of the mixtures. As controls and to determine the exact number of toxic units used, mice are inoculated with mixtures of equal parts of the same toxic suspensions and of the same dilutions of normal human or rabbit serum as those of the test sera. These control materials after standing at room temperature for 2 hours are further diluted 2-fold, 4-fold, and 8-fold in normal yolk and fluids and then are inoculated intravenously in 0.5 ml amounts.

2. *Neutralization with chicken antisera.* The second specific method lies in the use of chicken antisera. Unlike rabbit sera, which fail to give satisfactory neutralization of the infectious activity of the *Miyagawanellae*, sera prepared by intraperitoneal inoculation of the chicken give good neutralization and have a high specificity.[15]

*Immune serum preparation.** Following preimmunization bleeding, chickens are injected intraperitoneally 3 times a week with 3.0 ml. of 20 per cent saline suspensions of yolk sac or mouse lung heavily infected with *M. lymphogranulomatosis* or other desired agent of the group. Five days following the last injection, the chickens are bled. Injections are repeated until the serums have satisfactory neutralizing antibody titers. Fifteen injections have been found sufficient although very few injections may give satisfactory sera.

Determination of neutralizing titer of immune sera. Undiluted immune serum is mixed with equal volumes of serial 10-fold dilutions of heavily infected tissue beginning with a 1:5 dilution of tissue. Mixtures of infected tissue and normal serum should be included for control. Following incubation at 22° C. for 1 hour, 0.03 ml. of each of the mixtures is inoculated intranasally into six 7 to 10 gm. mice under light ether anesthesia.

Mice under test with *M. lymphogranulomatosis* should be observed for 5 or 6 days, 10 days' observation has proved more desirable for other members of the group. All mice that die, as well as those that are still living at the end of the observation period, are examined for pulmonary lesions. The serum titer is expressed numerically as the difference between the activity of normal and immune serum in terms of protection based either on mean infectivity score[15] or LD_{50}. The infectivity score appears to be the more satisfactory method of expressing the titer because of the greater reproducibility of the numerical value.

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* For this and the following description the author is indebted to Dr. Maurice R. Hilleman.

Neutralization by the intracerebral technic is likewise demonstrable for those viruses which infect by that route. Groups of six 15 to 20 gm mice are inoculated intracerebrally with 0.03 ml of the virus-serum mixtures described above and are observed for 21 days. Titer is expressed in terms of LD_{50} neutralized.

In a limited number of tests to date, using the intracerebral technic, definite neutralization has been demonstrated. The intranasal test appeared to be more satisfactory, however, in that a greater numerical value for neutralization was obtained. Highly quantitative and readily reproducible results are obtainable by the intranasal method.

Hyperimmune chicken sera also give agglutination of homologous elementary bodies as do rabbit antisera,[16] but the specificity of such agglutination reactions remains to be determined.

3. *Other characteristics.* Quite apart from the above methods are others by which the agents of the *Miyagawanelleae* may be distinguished. These depend on sulfonamide susceptibility and tissue tropisms.[3,4] *M. lymphogranulomatosis* is susceptible to sulfonamides as is also *M. bronchopneumoniae*[17] and some strains of *M. psittaci*.[18] *Chlamydium trachomatis* and *C. oculogenitale* are not considered since they have not been grown outside the human host. In tissue tropisms for example *M. psittaci* infects mice intraperitoneally and *M. lymphogranulomatosis* does not, *M. bronchopneumoniae* fails to infect mice intracerebrally although *M. lymphogranulomatosis* infects readily by this route.

F. Viability of Agent and Preservation

The agent is remarkably stable. It can be kept at 0° to 5° C. for periods up to weeks in pus, in yolk, or in less suitable fluids such as broth or even physiologic saline, with little if any loss of activity. At −72° C. the stability is much greater, and in yolk no change in activity has been detected in 3 months at this temperature. Storage at −32° C. is less satisfactory probably because of slow changes to and from frozen to thawed state which occur at this temperature. Dried from the frozen state the agent has remained viable in our experience for over 2 years with little change in activity.

III. IMMUNOLOGIC DIAGNOSIS OF THE DISEASE

A. Skin Test

The classical method of diagnosis of lymphogranuloma venereum depends on a sensitivity reaction, in the skin of infected persons, to an antigen containing inactivated agent. As originally described by Frei,[19] this was carried out with inactivated human pus. Later the use of in-

fected mouse brain was suggested.[20] Today the diagnostic material for the skin test is usually prepared from heavily infected yolk sacs.[21] The yolk sac antigen is standardized by prior testing on known positive and negative reactors and is diluted to give, in known positives, an intradermal reaction of suitable intensity. A control antigen is similarly prepared from normal yolk sacs.

Intradermal injections of 0.1 ml. amounts of both specific and normal antigens are made into the flexor surface of the forearm. The reaction is read in 48 hours, at which time the central induration or papule is measured, and the surrounding erythema is ignored. Provided that no indurated or papular response to the normal antigen occurs, a papule of 6 mm. or greater is significant.

The yolk sac antigen has proved more sensitive than that prepared from mouse brain in the detection of the disease. The skin test has proved most useful in diagnosis and in carrying out epidemiologic studies though it is less sensitive than the complement fixation test. Cross sensitivity in the skin test does occur in infections due to other *Miyagawanellae*[22] but has not been detected in other than these infections. A proportion of individuals are sensitive to normal yolk, however, and will react to this component in the antigen. The inclusion of a normal yolk control in the test should eliminate possible error due to such yolk sensitivity. Barwell [23] has shown that treatment of the agent with 0.02 normal HCl will extract an antigen which gives a specific skin sensitivity test, that is, without cross reaction infections due to other *Miyagawanellae*.

B. Complement Fixation Test

More recently the complement fixation test has been widely used for diagnosis of lymphogranuloma venereum.[24] In this case inactivated material from heavily infected mouse lung or yolk sac, preferably the yolk sac, is used. Other materials are not satisfactory because the concentration of specific antigen is too low.

In the earlier experiments infected yolk sac antigen and normal yolk sac control were treated with 0.1 per cent formalin in saline to inactivate the elementary bodies. Other methods of inactivation have been suggested, and the method finally recommended makes use of phenol and is based on the original work of Nigg.[25,26] It has the advantage of enhancing the activity of a given suspension of the agent and avoids, certainly to a large degree, those nonspecific reactions in early syphilis in which both specific and normal antigen give fixation,[27] and in which no diagnosis can be made. The method recommended by Nigg and Hilleman for preparing the antigen is as follows.[28]

A suspension of yolk sac membranes heavily infected with *M lymphogranulomatosis* is treated to contain 0.5 per cent phenol in a 10 per cent suspension of tissue Incubation of the suspension at 37° C for 3 weeks serves to inactivate the agent and to bring about a 16- to 32-fold enhancement of complement-fixing reactivity Heating fully enhanced phenolized antigen in boiling water for 20 to 30 minutes increases its avidity for lymphogranuloma venereum serum without change in titer

Inactivation of the agent is determined by inoculating 0 6 ml of a 1 5 dilution of the 10 per cent phenolized suspensions in the yolk sacs of twelve 6-day embryos The embryos are incubated for 12 days at 37° C Deaths occurring within the first 2 days after inoculation are considered nonspecific At least 10 of the 12 embryos must remain alive for 12 days after inoculation, and microscopic examination must rule out the presence of the agent

Control antigens are prepared in identical manner from normal yolk sacs of embryos of about the same age as the infected embryos

For use in the complement fixation test, stock commercial antigen prepared as above is diluted to give 1 unit in 0 2 ml , or, if prepared personally, is diluted so that it is not anticomplementary, gives definite reactions with known weakly positive sera, and gives no reaction with known normal sera Normal control antigen is diluted to the same extent

Guinea pig serum (fresh or frozen) is used as complement. Just before use it is titrated and diluted so that 2 units are contained in 0 2 ml This is usually obtained with 1 20 to 1 30 dilution

To reduce anticomplementary behavior of human sera to be tested, these are inactivated at 56° or 60° C for 20 minutes immediately before use Tests are set up as serum titrations and 2-fold dilutions of the sera in physiologic saline are prepared, starting at 1 2

The hemolytic indicator system consists of 0 2 ml of a 3 per cent suspension of washed sheep red cells sensitized for 30 minutes at 37° C with minimal hemolytic doses of antisheep cell rabbit amboceptor

The reagents in 0 2 ml amounts are added to a series of tubes in the following order diluted serum, 2 units of complement, specific antigen A control series is set up in each instance in which normal yolk sac material replaces the specific antigen, and suitable controls are also employed to exclude anticomplementary or hemolytic action of antigen or serum (at the highest concentration tested) as well as to demonstrate the activity of the complement on the hemolytic indicator

After incubation of 75 minutes in a 37° C water bath, 0 2 ml of the hemolytic indicator is added Readings for fixation (inhibition of hemolysis) are made after an additional incubation at 37° C for 30 minutes Only tests should be considered in which all controls are satisfactory

The test has proved a more sensitive diagnostic method than the intradermal test. That this is so can be shown in those cases which are observed from the earliest period after infection. Under these circum-

stances the complement fixation test becomes positive before the intradermal and, in event of successful therapy—and also, one would presume, in spontaneous cure—the intradermal test will become negative before the complement fixation reaction. It is not clear whether these two reactions depend on different antibody-antigen reactions or whether the differences merely represent a quantitative difference in threshold. What evidence there is points to the former [29,30]

Three main sources of error occur, two of which can be excluded by the use of the control antigen and one of which cannot. Sera from persons sensitive to certain constituents of normal yolk or yolk sac will react nonspecifically with both specific and control antigens. There is also a group of sera from syphilitic individuals in the early stages of the disease which shows a similar fixation with both antigens (see p. 462). The use of phenolized enhanced antigen avoids most if not all of the latter nonspecific reactions. In any case they disappear later in the course of the syphilis or following cure.

The third source of error occurs in individuals with latent or overt infection with other members of the family Chlamydaceae.[22,31] The predominant antigen is a group antigen which is common to all described members of the genera *Chlamydozoon* and *Miyagawanella*. Fixation with sera from such cases will be nonspecific within the group, but there will be no reaction with the normal yolk antigen At the moment methods so to purify the antigen complex as to remove the group antigen or antigens have been described only at the laboratory level and are not practical for routine use. Serum absorption studies have also been employed with steamed suspensions of elementary bodies but do not appear suitable for routine use. Hemagglutination-inhibition and indirect complement fixation tests have been described.[32] Neither, however, offers any practical advantages over the ordinary complement fixation test for the diagnosis of lymphogranuloma venereum in man.

Apart from these three sources of error the test is highly useful. Many cases of latent infection are uncovered, particularly in venereally infected persons, (i.e., with syphilis, gonorrhoea, or other venereal diseases) even among those whose intradermal tests are negative. Extensive epidemiologic studies are now possible so that sera collected for Wassermann, or for other serologic tests for syphilis, may be utilized for such surveys. It is not possible to say with certainty what level of serum dilution should be taken as the one beyond which a positive reaction indicates definite prior and continuing infection with some member of this group. Opinions differ on this point. We believe it to be significant

if fixation is obtained at dilutions of 1.4 or above. Mollaret[33] and others have stated that a certain proportion, about 50 per cent, of cases of cat scratch disease give a positive complement fixation test with antigen of lymphogranuloma venereum The significance of this observation is not clear although Mollaret believes it to be due to the fact that the etiologic agent of cat scratch disease belongs to the Chlamydaceae. Evidence on this point, however, is very slight

C Neutralization of Toxin

One other test on sera from cases of lymphogranuloma venereum has been used and may prove to have practical value. This is the specific neutralization of the toxic factor by antibodies in the serum. Little investigation of this point has been carried out, but what has been accomplished shows that such antibodies appear relatively soon after infection and are as specific as are the hyperimmune antibodies to the toxic factor produced in rabbits or chickens[14]

IV. REFERENCES

1 Moshkovskii, Sh D Tsitotropnie vozbyditeli infektsii i mesto rikkettsii v sisteme snlamydozoa *Usp Sovrem Biol*, 19 1–44, 1945

2 Harrop, G A, Rake, G, and Shaffer, M F Group of Laboratory Infections Ascribed to Lymphogranuloma Venereum *Tr Am Clin and Climatol A* (1940), 56 154–159, 1941

3 Shaffer, M F, Jones, H, Grace, A W, Hamre, D M, and Rake, G Use of the Yolk Sac of the Developing Chicken Embryo in the Isolation of the Agent of Lymphogranuloma Venereum *J Infect Dis*, 75 109–112, 1944

4 Wall, M J Isolation of the Virus of Lymphogranuloma Venereum from Twenty-eight Patients, Relative Value of the Use of Chick Embryos and Mice *J Immunol*, 54 59–64, 1946

5 Rake, G, and Jones, H Toxicologic and Therapeutic Action of Penicillin on Mice [illegible]

6 [illegible] 81 175–190, 1947

7 Sabin, A B, and Aring, C D Meningoencephalitis in Man Caused by Virus of Lymphogranuloma Venereum *J A M A*, 120 1376–1381, 1942

8 Rake, G., in Bergey's *Manual of Determinative Bacteriology* 6th ed. Baltimore The Williams and Wilkins Company, 1948, pp 1114–1120

9 Gonnert, R. Die Bronchopneumonie, eine neue Viruskrankheit der Maus *Zentralbl f Bakt Abt 1*, Orig 147 161–174, 1941

10 Yanamura, H C, and Meyer, K F. Studies on Virus of Psittacosis Cultivated *in Vitro J Infect Dis.*, 68 1–15, 1941

11 Rake, G, and Jones, H P Studies on Lymphogranuloma Venereum I. Development of Agent in Yolk Sac of Chicken Embryo *J Exper Med*, 75 323–338, 1942.

12 Pappenheimer, A M, Molloy, E., and Rose, H M. Presence of Granules Resembling Elementary Bodies in Yolk Cells of Normal Eggs. *Proc Soc Exper Biol and Med.*, 58 313–315, 1945

3 Complement fixation test
a. Antigen
b Positive control serum
c Complement fixation procedure
4 Hemagglutination-inhibition test

VII Exanthem Subitum

VIII Foot-and-Mouth Disease
A Introduction
B Laboratory Diagnosis
1 Virus isolation
2 Serodiagnosis
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IX Epidemic Viral Gastroenteritis

X Viral Hepatitis A and B
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XII Measles

XIII Molluscum Contagiosum
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1 Microscopic examination of material from the lesions
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XIV Mononucleosis, Infectious
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a Absorbing reagents
(1) Guinea pig kidney
(2) Beef erythrocytes
b. Absorption technic
c Interpretation of test results
3 Rapid test for heterophile antibody
a Evans test
b Tannen's and Glassy's tests
c Mason's test

I. ANEMIA, EQUINE INFECTIOUS (Swamp Fever of Horses, Equine "Malaria," Pernicious Anemia of Horses)

Equine infectious anemia is an acute or chronic febrile disease of horses characterized by progressive anemia, edema, and emaciation. It is rarely transmitted to man.[1] Equine species are susceptible to the disease; ordinary laboratory animals are not.[1,2] Laboratory diagnosis[3] of the illness in man is accomplished by the demonstration of the typical disease in horses after subcutaneous injection of 25 ml. or more of filtered serum from the patient taken during the acute phase of the illness. The test horses should be observed for at least 60 days in an iso-

3 Complement fixation test
a Antigen
b Positive control serum
c Complement fixation procedure
4 Hemagglutination-inhibition test

VII EXANTHEM SUBITUM

VIII FOOT-AND-MOUTH DISEASE
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IX EPIDEMIC VIRAL GASTROENTERITIS

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2. Absorption test to determine type of heterophile agglutinin
a Absorbing reagents
(1) Guinea pig kidney
(2) Beef erythrocytes
b Absorption technic
c Interpretation of test results
3. Rapid test for heterophile antibody
a. Evans test
b Tannen's and Glassy's tests
c Mason's test

Intradermal injection of 0 1 ml of antigen gives rise in 48 hours to a papule 0.5 to 1 0 cm in diameter or an area of erythema 1.5 to 6.0 cm in diameter or both. Nearly all patients with cat scratch disease exhibit a positive test Frei reactions in these same patients are negative

B Skin Test Antigen for Clinical Diagnosis

Skin test antigen is prepared from human material [1,6] The affected node is allowed to proceed to suppuration and the pus is aspirated aseptically. In the absence of pus, macerated necrotic lymph node tissue may also function satisfactorily. The material is triturated with sufficient (1 part tissue to 1, 2, 3, or 4 parts diluent) sterile physiologic saline solution to give a homogeneous emulsion and is heated to 56° C. for 1 hour on 2 consecutive days[1] or at 60° C for 2 hours on 1 day and 1 hour on the day following [6] Sterility tests are performed under aerobic and anerobic conditions to detect the presence of viable bacteria or fungi As a further precaution, the patients supplying material should be carefully selected to exclude tuberculosis, infectious hepatitis, syphilis, lymphogranuloma venereum, and other transmissible maladies It also seems advisable to add a preservative (for example, merthiolate in 1 10,000 final concentration) to the antigen

The potency of the antigen is determined by skin tests in patients with the disease Certain preparations may be sufficiently potent to permit use in diluted form, this may be decided after intradermal titration of serial 2-fold dilutions of the material in patients known to react in a positive fashion with cat scratch skin test antigen The standardized antigen must not react in "normal" control persons. This antigen is not available commercially

C Laboratory Diagnosis

1 *Complement fixation test* Complement fixation tests with psittacosis-lymphogranuloma venereum group antigens should be performed with sera from the patient collected at the onset of illness and 2, 4, and 8 weeks later. Antigen may be prepared in the individual laboratories[13] or purchased from commercial sources. The findings must be evaluated with caution, as discussed in section II, A above.

III COMMON COLD (Acute Coryza, Acute Rhinitis)

The common cold is an infection of the upper respiratory tract caused by a viral agent Colds themselves are generally mild and of

lated, screened stable, and the temperatures should be observed twice daily.

II CAT SCRATCH DISEASE (Cat Scratch Fever, Benign Lymphoreticulosis of Inoculation), Cat Scratch Encephalitis, and Parinaud's Oculoglandular Syndrome

A. Introduction

Cat scratch disease was recognized as early as 1932 by Dr. Lee Foshay,[1] but the first published description of the illness was given by Debré and his associates in 1950[2] Since 1950, there have been more than 100 reported cases of this disease[3-8]

Cat scratch disease is a self-limited and nonfatal systemic illness of common occurrence which is generally recognized in persons who have been scratched by a cat The illness is characterized by fever, malaise, and lymphadenitis which is often preceded by a cutaneous lesion The infected lymph nodes may proceed to suppuration, and an erythematous maculopapular eruption or measles-like rash may also be present Rarely, central nervous system disease, termed cat scratch encephalitis,[9] with convulsions, respiratory paralysis, and coma, may occur. Present evidence, based on positive skin sensitivity reactions with cat scratch disease antigen,[10] suggests that Parinaud's oculoglandular syndrome may be caused, in certain instances, by the agent of cat scratch disease

The etiology of cat scratch disease was investigated by Mollaret and his associates[11,12] who reported successful transmission of the disease to monkeys by [illegible] fect other laboratory animals were unsuccessful.

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Skin test antigen is prepared from human material [1,6] The affected node is allowed to proceed to suppuration and the pus is aspirated aseptically In the absence of pus, macerated necrotic lymph node tissue may also function satisfactorily. The material is triturated with sufficient (1 part tissue to 1, 2, 3, or 4 parts diluent) sterile physiologic saline solution to give a homogeneous emulsion and is heated to 56° C. for 1 hour on 2 consecutive days[1] or at 60° C for 2 hours on 1 day and 1 hour on the day following.[6] Sterility tests are performed under aerobic and anerobic conditions to detect the presence of viable bacteria or fungi As a further precaution, the patients supplying material should be carefully selected to exclude tuberculosis, infectious hepatitis, syphilis, lymphogranuloma venereum, and other transmissible maladies. It also seems advisable to add a preservative (for example, merthiolate in 1 10,000 final concentration) to the antigen

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lated, screened stable, and the temperatures should be observed twice daily.

II CAT SCRATCH DISEASE (Cat Scratch Fever, Benign Lymphoreticulosis of Inoculation), Cat Scratch Encephalitis, and Parinaud's Oculoglandular Syndrome

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The agent of CTF was first isolated in 1944 by Florio and his colleagues,[4] who succeeded in transmitting the infection to hamsters inoculated with serum from a patient with the disease Since that time,[5] additional strains have been recovered in suckling mice The virus may be propagated in embryonated eggs[6] inoculated via the yolk sac The mouse-adapted strain produces a viremia after intracerebral inoculation in mice, cotton rats, hamsters, and opossums but not in sheep or rabbits[7]

B. Laboratory Diagnosis

Laboratory diagnosis is accomplished either by isolation and identification of the causal agent or by the demonstration of a significant increase in amount of CTF antibody in the patient's serum during convalescence The procedures regularly employed at the Walter Reed Army Institute of Research are described below

1 *Virus isolation* Serum or whole blood clot collected from the patient during the acute illness is inoculated intraperitoneally in 0 1 ml. amount and intracerebrally in 0 01 ml volume into baby mice 3 to 5 days old Baby mice are more susceptible to CTF virus of human origin than are adult mice or hamsters and, for this reason, are the animal of choice for recovery of virus[5] Infected mice become excitable and hyperirritable and show signs of muscular inco-ordination which is followed by terminal stupor and death in 48 hours Mice failing to show signs of illness on primary passage should be sacrificed on the 6th to 10th day after inoculation and a 10 per cent suspension of their brains passed to new mice The recovered strains may be identified with known positive serum in the serum neutralization or complement fixation test.

2 *Serum neutralization test*

a Seed virus Twelve to 14 gm white mice are inoculated intracerebrally with approximately 1,000 LD_{50} (0 03 ml. inoculum dose) of the mouse-adapted Florio strain of CTF virus The brains are harvested when 50 per cent or more of the mice show signs of advanced central nervous system disease and are emulsified with 30 per cent normal rabbit serum-saline solution to give a 20 per cent suspension This material is centrifuged at 2,000 r p m for 10 minutes and the supernate is the seed virus The virus is preserved by storage at $-70°$ C in glass-sealed ampules, and the preparation should titer around 10^{-7} when tested in adult mice inoculated intracerebrally.

b Positive control serum At weekly intervals young adult rabbits are given 3 intraperitoneal injections of 5 0 ml. amounts of the seed virus preparation described above and are bled on the 10th day after

short duration but may be followed by secondary bacterial infection of a more serious nature.

There is no specific laboratory diagnosis for the common cold. The disease may be transmitted to human volunteers[1,2] or to the chimpanzee,[3] using bacteria-free filtrates from nasal washings of patients with colds. Successful propagation of the agent in tissue culture of chick embryo[4] and on the chorioallantoic membrane[5] of embryonated eggs has been reported. Several papers have described cultivation of the virus in the allantoic sac of the chick embryo,[6-8] but Andrewes and his associates[9] at the Common Cold Research Unit in Salisbury, England, were unable to repeat these findings. More recently, Andrewes *et al*[9] announced propagation of a common cold agent through 10 serial passages of human lung tissue culture media. The infected tissue culture cells failed to show a cytopathogenic effect, and the procedure was one of little practical value since viral growth could only be proved by tests in human volunteers

Viruses of the RI* family,[10,11] also called the APC family[12] including agents designated ARD[13], cause such acute respiratory illnesses of man as undifferentiated acute respiratory disease (ARD), exudative pharyngitis, pharyngoconjunctival fever, bronchitis resembling atypical pneumonia, and atypical pneumonia unassociated with the development of cold or streptococcus MG agglutinins These viruses may also cause illness that might be classed as "severe cold." These agents, however, do not appear to be related etiologically to the "typical" common cold, which is characterized by coryza, profuse watery discharge, and a mild febrile or afebrile course of short duration. Information concerning these viruses is given in the chapter on "Primary Atypical Pneumonia."

IV. COLORADO TICK FEVER (Tick Fever, Mountain Fever, Mountain Tick Fever, American Mountain Fever, Nonexanthematous Fever)

A. Introduction

Colorado tick fever (CTF) is a nonexanthematous and nonlethal disease of man which is transmitted by the wood tick, *Dermacentor andersoni*[1-3] The illness is characterized by sudden onset, fever, headache, backache, and deep ocular pain Nausea and vomiting, photophobia and hyperesthesia of the skin may also be present The illness is usually intermittent, consisting of 2 or 3 bouts of symptoms lasting 2 to 4 days and separated by relatively asymptomatic periods of 2 or 3 days' duration In typical cases, there is a moderate leucopenia, with white cell counts of 2,000 to 3,000 per cmm

* These viruses have recently been named the adenoviruses

terial should be accomplished by introduction of distilled water with a syringe through the diaphragm of a suitable rubber closure.

b Positive control serum Fourteen to 16 gm white mice are injected intraperitoneally at weekly intervals with 0 5 ml amounts of a 10^{-1} dilution of the 10 per cent mouse brain seed virus preparation used for the serum neutralization test (see above) The mice are bled 10 days after the last virus injection

c. Complement fixation procedure Specimens of serum collected from the patient at onset of illness and 14 to 21 days later are tested simultaneously by the C F method To serial 2-fold dilutions of the inactivated serum (56° C 30 minutes) in the range from 1 5 to 1 320 are added 2 full units of guinea pig complement in 0 5 ml and 4 units of viral antigen in 0 25 ml or 0 25 ml of appropriately diluted normal control antigen After overnight fixation at 4° C, 0 5 ml of the hemolytic system (0 5 ml of 1 5 per cent sheep cell suspension containing 3 *units of sheep cell amboceptor) is added, and the tests are read after* final incubation at 37° C for 30 minutes The titer is the highest initial dilution of serum which gives 3 + or greater fixation of complement As with C F tests in other diseases, a 4-fold or greater increase in antibody level in the patient's serum during convalescence is diagnostic and is of greater significance than is a positive test with a single specimen alone Sera from persons with syphilis may react with the mouse brain component of the antigens, such reactivity may be reduced by inactivating the serum at 60° C or 65° C instead of at the usual 56° C temperature[9]

V ECTHYMA CONTAGIOUS (Ovine Pustular Dermatitis, Orf, Contagious Pustular Dermatitis of Sheep Scabby Mouth)

A Introduction

Contagious ecthyma is a viral disease of sheep and goats characterized by the development of vesiculopustular lesions on the lips and adjacent tissues and the nonwool-bearing parts of the body The infection is transmissible to human beings[1-4] and occurs in persons handling infected sheep In man, the disease is mild and self-limited and is characterized by the formation of papules and papulovesicular and pustular lesions on the hands and face

Pask *et al*[3] reported successful transmission of the C.S L strain of contagious ecthyma to human volunteers, using bacteriologically sterile filtrates of material obtained from the lesions of infected sheep The virus was recovered in lambs inoculated with scrapings from the human lesions, and 11 of 12 persons convalescent from the experimental disease were solidly immune to reinfection.

the last injection of virus. The serum should neutralize at least 1,000 LD_{50} of virus in the intracerebral test in mice.

c. Serum neutralization procedure. Sera collected from the patient at onset of illness and 3 or 4 weeks later are tested simultaneously by the neutralization method. Two-tenths ml. amounts of the serum (uninactivated*) to be tested are mixed with 0 2 ml. volumes of serial 10-fold dilutions of virus. After incubation at 37° C. for 1 hour, groups of six 8-10 gm. mice are inoculated intracerebrally with 0 03 ml. amounts of the serum virus mixtures The acute phase serum is tested with 0, 1, 10, and 100 LD_{50} of CTF virus, and the convalescent specimen is tested with 10, 100, 1,000, and 10,000 LD_{50} of virus. Known negative and positive control sera are included in the tests. The mice are observed daily for 14 days for development of central nervous system illness, and the deaths are recorded. In a positive test, the convalescent serum specimen neutralizes 100 more LD_{50} of virus than does the acute phase sample.

3 *Complement fixation test.*

a Antigen. Twelve to 14 gm. white mice are inoculated intracerebrally or suckling mice (4 to 5 days old) are injected by the intraperitoneal route with approximately 1,000 LD_{50} of virus, and the brains are harvested from animals which are prostrate or dead for only a short period of time. The brains are emulsified in a mortar with physiologic saline solution or shaken with glass beads to give a 20 per cent suspension The suspension is centrifuged at 2,000 r p.m. for 10 minutes to remove large particles, and the supernate is clarified by spinning at 13,000 r p m. for 1 hour in an angle-head machine. The supernate is the antigen, and the preparation usually gives significant fixation of complement in a final dilution of 1.8 or 1·16 of brain tissue This antigen is infectious so that due precaution should be exercised to prevent infection

be employed.[8] Antigens for control purposes are prepared in identical manner from brains of uninoculated mice. If benzene extraction is to be employed with dried live virus preparations, then due precaution must be taken to prevent the powder from getting into the air. Drying is best accomplished by lyophilization, and rehydration of the dehydrated ma-

* Some workers prefer to inactivate the sera at 56° C for 30 minutes

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The relationship between contagious ecthyma virus and the pox group of agents has not been fully determined. The elementary bodies seen in smears of lesions from human cases of contagious ecthyma or smallpox stained by Gutstein's or Paschen's method are indistinguishable[4,5] Abdussalem (quoted in Reference 5), however, reported that even though both viruses are brick-shaped when examined in the electron microscope, they nevertheless can be distinguished morphologically. Contagious ecthyma and vaccinia viruses are immunologically distinct when compared in complement fixation tests with convalescent sera from human beings infected with these agents[5] Further, vaccinia but not contagious ecthyma virus can be propagated on the chorioallantoic membrane of the embryonated egg.[3-5]

B. Laboratory Diagnosis

1. *Virus isolation.* There is no standardized technic for recovery and identification of the virus from human cases of contagious ecthyma. Giltner[6] suggested, however, that the following procedure be used:

Pustular material from the lesions or the dried scabs are emulsified with hormone broth and rubbed into the scarified glabrous skin of the inner thigh or postventral abdomen of lambs In positive cases, erythematous patches which become vesicular and finally pustular appear in 3 to 10 days, the incubation period depending upon the amount of viable virus present in the original inoculum. The pustules usually rupture in 24 to 48 hours and form thick, brownish scabs; healing is complete in 25 days. Finally, the recovered virus is identified by simultaneous inoculation of 2 immunized lambs (at least 2 weeks' postvaccination*) and 2 control lambs. The vaccinated animals should show only a slight or no reaction, and the control lambs should develop the typical lesions. Bacterial etiology is ruled out by cultivation attempts on suitable media.

2. *Serodiagnosis.* MacDonald[5] reported positive complement fixation results (serum titer 1:4 to 1:32) in sera from 9 of 20 cases of contagious ecthyma infection in man The antigen used in the tests consisted of a clarified suspension prepared from the vesicular eruptions of sheep infected experimentally with a strain of virus obtained originally from a human case of the disease Unfortunately, the acute and convalescent sera from the patients were not compared, so that it is difficult to assess the significance of these results. Blakemore *et al.*[7] reported that sera from patients with the disease agglutinated washed elementary body suspensions of contagious ecthyma virus.

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* Vaccine is available from several commercial sources

VI. ENCEPHALOMYOCARDITIS (Columbia-SK Disease, MM-Virus Infection, Mengo Encephalomyelitis)

A Introduction

The encephalomyocarditis (EMC) family of viruses comprises a group of closely related agents which includes the Columbia-SK, MM, EMC, Mengo encephalomyelitis, F, and Ortlieb strains[1,2] These agents are highly pathogenic for rodents (mice, cotton rats, hamsters, guinea pigs) inoculated by the cerebral or peripheral routes, causing a fatal encephalitis which may be accompanied by a myocarditis Infected monkeys develop a poliomyelitis-like disease and often recover Strains of EMC virus have been isolated from monkeys, man, mosquitoes, and the mongoose infected in nature Antibody to the agent is commonly present in the sera of wild-caught rats,[3] and these animals may constitute a reservoir for the disease

Proved human EMC infections are few Smadel and Warren[4] demonstrated increase in neutralizing antibody to the EMC virus in paired sera from patients who suffered from a mild febrile disease called "3 day fever," which appeared in 1945-46 among U.S Army troops in the Philippines The patients presented clinical findings of severe headache, moderately high fever of 2 to 3 days' duration, pharyngitis, stiff neck, positive Kernig's sign, and hyperactive deep reflexes The only notable laboratory finding was pleocytosis from 50 to 500 cells, principally lymphocytes, in the spinal fluid A case of acute encephalitis[5] occurred in a laboratory worker who was handling the Mengo strain, the virus was recovered from the patient, and an increase in amount of neutralizing antibody was demonstrated in the patient's serum after recovery from the illness More recently (quoted in Reference 2) strains of EMC virus were recovered in West Germany from patients with various symptoms which included encephalomyelitis and myocarditis, one of the cases terminated fatally

B Laboratory Diagnosis

1. *Virus isolation* Little is known about the distribution of the virus in human tissues Virus isolation, however, should be attempted by intracerebral inoculation of white mice with spinal fluid or serum collected from the patient during the acute disease or with clarified suspensions of brain, cord, myocardium, and spleen from fatal cases. Mice inoculated with small amounts of a neurotropic laboratory-adapted strain of the virus become lethargic, develop a flaccid paralysis, and die in 3 or 4 days, given a large dose of virus, acute encephalitis develops without paralysis, and death occurs in 18 to 24 hours. On primary isolation the incubation period is considerably longer Virus recovered from the patient is identified in neutralization tests with known positive sera by the general method described below

2. *Serum neutralization test.* Serum neutralization and complement

fixation tests are performed with sera from patients collected at onset of illness and again 3 or 4 weeks later, and the acute and convalescent specimens are always titrated simultaneously in the same test. The serum neutralization and complement fixation procedures described below are those routinely employed in the Diagnostic Laboratory of the Department of Virus Diseases, Walter Reed Army Institute of Research.

a. Seed virus This consists of the low speed supernate of a 20 per cent suspension of brains harvested from inoculated mice showing symptoms of advanced EMC infection; brains from dead mice are not included The brain suspension is prepared in 30 per cent normal rabbit serum saline solution and is preserved by storage at -70° C. in the dry ice refrigerator The LD_{50} titer of such seed virus preparations is usually $10^{-8.5}$ in intracerebral mouse tests or 10^{-7} when the mouse intraperitoneal test is used

b. Positive control serum for use in the diagnostic neutralization test and for identification of newly recovered strains. Young adult rabbits are hyperimmunized by 3 intraperitoneal injections at weekly intervals of 5.0 ml. amounts of the seed virus preparation described above. The rabbits are bled 10 days after the last injection, and the sera should neutralize 100,000 LD_{50} of EMC virus when tested in mice inoculated intraperitoneally or 1,000 LD_{50} by the mouse intracerebral test.

c. Serum neutralization procedure. Four-tenths ml. amounts of the serum (not inactivated*) to be tested are mixed with 0.4 ml. volumes of the proper serial 10-fold dilutions of seed virus and, after incubation at 37° C for 1 hour, 0.1 ml. amounts of the mixture are inoculated intraperitoneally into groups of 6 mice weighing 12 to 14 gm. Acute phase patient's serum is tested with 1, 10, 100, and 1,000 LD_{50} of virus and the convalescent serum with 10, 100, 1,000, and 10,000 LD_{50} of the agent. A known positive control serum is included in the tests, and a titration of the seed virus is performed simultaneously. The mice are observed for the development of typical central nervous system signs for 14 days, and the deaths are recorded The LD_{50} titers are calculated according to the Reed and Muench formula,[6] and a positive test with paired sera from a patient is one in which the convalescent specimen neutralizes at least 100 LD_{50} more virus than does the acute phase sample.

* Some workers prefer to inactivate the serum at 56° C for 30 minutes

3. *Complement fixation test.*

a. Antigen A 20 per cent suspension of EMC-infected mouse brain in physiologic saline solution is centrifuged for 10 minutes at 3,000 r p m. in the horizontal machine to remove large particles, and the supernate is clarified by spinning for 1 hour at 13,000 r p.m in the angle-head centrifuge The water-clear supernatant fluid is the antigen and, since it is infectious, the usual precautions should be taken The potency is preserved for several months when stored at –20° C. or at –70° C. in glass-sealed vials Control antigen is prepared in an identical manner from brains of normal mice of the same age

b Positive control serum Convalescent sera from EMC-infected rats, hamsters, mice, or monkeys are employed The animals are injected peripherally with sublethal amounts of virus grown in the same host and are bled after recovery from the illness (see Reference 7 for further details)

c Complement fixation procedure The paired sera from the patient are tested simultaneously with EMC or normal mouse brain antigen by the same C F technic described for diagnosis of Colorado tick fever (see IV, B, 3, c above) A positive diagnostic test with the paired sera from a patient is one in which the titer of the convalescent serum specimen is at least 4 times higher than that of the acute phase sample.

4 *Hemagglutination-inhibition test* Viruses of the encephalomyocarditis group cause agglutination of sheep erythrocytes in the cold (5° C) but not at room temperature,[8,11] and this hemagglutination reaction is inhibited by specific antibody Tests to measure antibody in serum specimens may be carried out by the same general hemagglutination-inhibition method employed in influenza virus studies.[12,13] A clarified suspension of brains of mice infected with a virus of the EMC group serves as hemagglutinating antigen in the test The hemagglutination-inhibition technic has not been evaluated as a diagnostic tool in tests with sera from human cases of EMC infection It should be noted, however, that a high percentage (27 per cent) of sera from patients with central nervous system disease (paralytic or nonparalytic poliomyelitis, aseptic meningitis, or encephalitis) gave positive reactions in an EMC hemagglutination-inhibition test devised by Gard and Heller.[11] None of these same sera neutralized EMC virus in animal experiments, and the positive hemagglutination-inhibition findings may have been due to nonspecific factors in the test.

VII. EXANTHEM SUBITUM (*Roseola Subitum, Roseola Infantum,* Roseola Infantalis, Pseudo-Rubella, Rose Rash of Infants)

Exanthem subitum[1] is an acute illness with sudden onset which affects infants and young children primarily and is characterized by fever of from 3 to 5 days' duration. It is followed by the development of a transient macular or maculopapular rash, which appears on the neck, trunk, upper extremities, thighs, and buttocks about the time that the fever subsides. Desquamation is rare, pigmentation does not occur, and an exanthem of erythematous specks and streaks may occur on the soft palate. Meningismus may be present occasionally, and leucopenia and relative or absolute lymphocytosis are characteristic, being most marked around the 3d day of fever. Slight enlargement of the lymph nodes may be present.

The disease is believed to be of viral etiology, and the agent apparently may produce a febrile illness in monkeys.[2] Neva and Enders[3] recently reported the recovery of a cytopathogenic agent in human cell tissue cultures from the stool of a patient with illness resembling roseola infantum. Development of neutralizing and complement-fixing antibodies against the agent was demonstrated in the patient's serum during convalescence. Rowe *et al.*[4] found this agent to be a type 3 virus of the family of viruses commonly referred to as the RI,*[5,6] APC,[4] or ARD[7] agents. Additionally, Neva and Enders[8] recovered a different group of "atypical viruses" in human cell tissue cultures from the stools of patients with an unusual epidemic exanthem.

VIII. FOOT-AND-MOUTH DISEASE (Fièvre Aphteuse, Maul-und-Klauenseuche, Aphthous Fever, Epizootic Stomatitis)

A. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-footed animals, especially cattle, sheep, goats, and pigs (for general information see References 1 and 2). The disease in these animals is characterized by fever, increased salivation, and the appearance of fluid-filled vesicular lesions on the mucous membranes of the mouth, tongue, and lips, on the muzzle, between the claws, and on the teats and udder. The disease is occasionally transmitted to man, causing a self-limited febrile illness characterized by pain in the limbs, excessive salivation, and the appearance of vesicular lesions on the buccal or lingual epithelium and on the skin of the hands, feet, and other parts of the body.

FMD is caused by a group of biologically similar viruses which are differen-

* These viruses have recently been named the adenoviruses.

tiated into 6 immunologic types These are the A, O, and B types of Waldmann and [illegible] disease may be transmitted readily to guinea pigs or suckling mice,[3] whereas rabbits, rats, adult mice, dogs, and cats are irregularly susceptible Fowl, ferrets, and horses are usually resistant

Owing to the rigid quarantine procedures by the Bureau of Animal Diseases, U.S Department of Agriculture, the United States is usually free of FMD Further, public law[5] prevents the importation of the virus into the mainland of the United States, the Secretary of Agriculture may, however, establish laboratories for research on FMD on islands separated from the mainland by deep navigable streams.

B Laboratory Diagnosis

FMD is diagnosed either by recovery and identification of the virus from the vesicular fluid obtained from lesions in the patient or by demonstration of increased amount of antibody in the patient's serum during convalescence from the illness

1 *Virus isolation* Young adult guinea pigs inoculated intradermally into the foot pads develop vesicular lesions at the site of injection in 24 to 48 hours, and this is followed by the appearance of vesicles in the mouth, 18 to 36 hours later The disease is usually nonlethal in guinea pigs, and the virus can be demonstrated in the blood at the time of appearance of the initial lesions Suckling mice (7 to 14 days old) inoculated intracerebrally develop spastic muscular paralysis of the hind limbs 1 to 2 days after injection, with spread to the remaining part of the body and death within 12 hours after the initial illness The recovered virus is identified by pathogenicity tests in animals and by cross-immunity or serologic procedures

FMD in the natural hosts is clinically indistinguishable from 2 other vesicular diseases which occur in the United States These are vesicular stomatitis of horses and cattle and occasionally man[6] and vesicular exanthema of swine. For preliminary differentiation of these 3 agents, Giltner[7] recommends passage of the infectious material intradermally onto the tongue of the horse and cow, intramuscularly into the cow, and intradermally into the snout of the pig. Based on the development of vesicular disease in these animals, as summarized in Table 1, preliminary differentiation can be made.

Further identification and strain typing is accomplished by the serum

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IX. GASTROENTERITIS, EPIDEMIC VIRAL (Epidemic Diarrhea, Acute Infectious Gastroenteritis, Epidemic Diarrhea of the Newborn, Stomatitis and Diarrhea of Infants)

Gastroenteritis of nonbacterial etiology[1-4] may occur in sporadic or epidemic form during any season of the year. The disease affects persons of all ages and may induce chills, fever, malaise, muscular aches and pains, dizziness, anorexia, vomiting, gastric pain, and diarrhea. Stomatitis may be present in at least one form of the illness. The disease may be lethal, especially in the aged or infirm and in infants.

Information on the etiology of epidemic gastroenteritis remains in the developmental stages. Reimann *et al.*[1,5] reported transmission of the disease to human volunteers by the respiratory but not the alimentary route, using filtered throat washings or fecal suspensions from patients suffering with the illness. Gordon *et al.*[6] transmitted the infection to human volunteers by oral administration of fecal filtrates or unfiltered throat washings from patients with the disease, illness failed to develop in persons who inhaled throat washings from the patients. More recently, Jordan *et al.*,[7] working with human volunteers, distinguished between 2 agents of gastroenteritis based on differences in incubation period, the clinical picture, and the absence of cross-immunity.

Two viral agents have been recovered in experimental animals inoculated with materials from cases of the infantile disease. Light and Hodes[8] produced a transmissible bloody mucoid diarrhea in calves inoculated nasally with filtered stools from cases of infantile diarrhea. They were unable to recover the agent from the stools of normal infants or normal calves, and calves recovered from the experimental disease were immune to reinfection. These results await confirmation by other groups, especially since a number of natural diarrheal diseases of calves have been described[9,10] in recent years. Buddingh and Dodd[11,12] studied epidemics of diarrhea of the newborn in which stomatitis was a prominent feature of the illness. A transmissible filtrable agent was recovered from oral or fecal swabs from sick infants which caused haziness or cloudiness on the scarified rabbit cornea within 24 hours after inoculation. Marked hyperemia and swelling of the palpebral conjunctivae was also present. Infantile diarrhea patients developed neutralizing antibody for the agent during recovery from the disease. This same viral agent was recovered from the genital tract of the normal adult human female and from a male patient with Reiter's disease.[11,12] It was suggested that the agent may

TABLE 1

DIFFERENTIATION OF VESICULAR DISEASE AGENTS BY EXPERIMENTAL INOCULATION INTO DOMESTIC ANIMALS

Animal	Inoculation Route	Result after inoculation of the virus of		
		FMD	Vesicular Stomatitis	Vesicular Exanthema
Horse	Intradermal	0	+	±
Cow	Intradermal	+	+	0
	Intramuscular	+	0	0
Pig	Intradermal	+	+	+

neutralization or the complement fixation procedure referred to in the following paragraph.

2. Serodiagnosis

a. Serum neutralization test. The acute and convalescent (2 to 3 weeks after onset of illness) sera from the patient are tested by the usual neutralization procedure with representative viruses of the 6 basic immunologic types A convenient source of virus for such tests is vesicular fluid from infected guinea pigs or infected brain tissue from suckling mice Positive control antisera for use in the tests are obtained from guinea pigs which are convalescent from the disease and which may be hyperimmunized by repeated intramuscular injections of the same infecting agent. The neutralization tests may be carried out using guinea pigs inoculated intradermally into the foot pads, baby mice inoculated intracerebrally, or by inoculation into the tongues of cattle[3,8,9] A positive test is one in which the convalescent serum from the patient neutralizes at least 100 more LD_{50} of virus than does the acute phase specimen

b. Complement fixation test Several variations[8,10-12] of the conventional complement fixation test procedure have been described. Extracts of tongue epithelium from cattle infected with viral strains representative of the different immunologic types usually serve as antigen in these tests. Suspensions of infected suckling mouse brain[3] or virus propagated in mass tissue culture explants of bovine tongue epithelium[13] should prove equally satisfactory. The demonstration of a 4-fold or greater increase in antibody in the patient's serum for FMD virus during convalescence constitutes a positive test

promising but are still in the developmental stage Specific complement fixation reactions have been reported in tests employing sera from patients with infectious hepatitis,[6] serum hepatitis[6,7] or post-arsphenamine hepatitis,[8] and antigens prepared from tissues of patients with these illnesses, but no procedure of practical value has yet been developed

XI. KERATOCONJUNCTIVITIS, EPIDEMIC (Shipyard Conjunctivitis, Superficial Punctate Keratitis, Epidemic Infectious Conjunctivitis, Keratitis Maculosa)

A. Introduction

Epidemic keratoconjunctivitis (EK) is a viral disease characterized by a nonpurulent inflammation of the conjunctivae, preauricular lymphadenitis, and superficial punctate keratitis. The disease is self-limited although keratitis with visual impairment may persist

The illness first assumed major importance in the United States when it occurred at a Veterans Administration hospital in California in 1936 [1] It later emerged as an epidemic in the shipyards and came to be known as "shipyard conjunctivitis."[2]

The viral etiology of EK was first reported by Sanders and Alexander[3] in 1943. These workers apparently recovered a filtrable agent from patients with the disease which could be propagated in cultures of minced chick embryo[3] and in the allantoic cavity of the embryonated egg [4] Adult mice inoculated intranasally or intracerebrally with the virus became lethargic within 2 to 7 days, this was followed by convulsions, spastic paralysis, and death within 24 hours after onset of illness. Rabbits developed a fatal encephalitis within 8 days after intracerebral injection of the agent and irregularly showed a herpes-like keratitis after inoculation of the virus onto the scarified cornea, acidophilic intranuclear inclusions could be demonstrated in the corneal sections

Maumenee *et al*[5] showed that the EK virus was antigenically related to but not identical with the virus of herpes simplex, and it was believed that the 2 agents should be considered separate entities More recently, Ruchman[6,7] and Cheever[8] showed a close relationship between St Louis encephalitis virus and an agent which now bears the label of EK agent The 2 viruses were indistinguishable serologically[6,7,8] and showed the same age-susceptibility pattern in rats[9] The viruses differed, however, in that the EK but not the St. Louis encephalitis strain was capable of initiating a fatal encephalitis in guinea pigs and rabbits.[7] Whether the agent now labeled EK is indeed the same virus isolated from the epidemic keratoconjunctivitis patient or whether it is a laboratory contaminant inadvertently introduced into the culture during the course of animal passage remains to be clarified. More recently, Jawetz *et al*[10] recovered a virus from cases of epidemic keratoconjunctivitis which was found[11] to belong to the family of viruses known as RI*[12,13], APC[11,14], or ARD[15] agents It is to be noted also that conjunctivitis without keratitis may be a prominent feature among patients with respiratory illnesses caused by viruses of this general group[16,17]

* These viruses have recently been named the adenoviruses.

be transmitted venereally in adults and that the infant may contract the disease during passage through the birth canal. These observations are of considerable interest and have for a number of years awaited confirmation by other groups of workers.

No satisfactory laboratory tests for epidemic gastroenteritis have been developed, however, attempts should be made to recover and characterize the responsible agents in future epidemics. Keitel[13] reported that an appreciable proportion of infants with gastroenteritis may develop cold and streptococcus MG agglutinins in the course of their illness

X. HEPATITIS A, VIRAL (Infectious Hepatitis, Epidemic Jaundice) and HEPATITIS B, VIRAL (Serum Hepatitis, Homologous Serum Jaundice, Transfusion Jaundice)

A. Introduction

Viral hepatitis A (for review see References 1 and 2) is a subacute viral infection of world-wide distribution which occurs in endemic or epidemic form. The illness is characterized by fever, anorexia, nausea, vomiting, abdominal distress, diffuse involvement of the liver, and usually jaundice. Outbreaks of the disease may be explosive or sporadic, and the epidemics commonly occur in the fall or early winter, with decline during the spring. The infection seems to be spread by the intestinal-oral circuit, and water, milk, and food-borne epidemics have been described.

Viral hepatitis B (for review see References 1 and 2) is clinically indistinguishable from viral hepatitis A. The disease is transmitted from person to person through the medium of improperly sterilized hypodermic needles or syringes or by the injection of certain human blood products and other biologicals such as vaccines containing human serum or plasma Human beings are apparently insusceptible to the virus given orally, and the agent has not been demonstrated in the feces of patients with the disease, thus the intestinal-oral circuit appears not to be of importance in the transmission of this infection. The viruses of hepatitis A and B are said to be immunologically distinct.

B. *Laboratory Diagnosis*

Liver function tests may assist materially in establishing the existence of a hepatitis. However, there are no specific laboratory tests of proved reliability for these diseases Henle *et al.*[3] reported propagation of hepatitis virus A in tissue cultures of chick embryos and in the embryonated hen's eggs. The amniotic fluid from infected eggs, irradiated with ultraviolet light, was found to give positive skin tests in patients known to have suffered from infectious hepatitis.[4,5] These findings are highly

promising but are still in the developmental stage. Specific complement fixation reactions have been reported in tests employing sera from patients with infectious hepatitis,[6] serum hepatitis[6,7] or post-arsphenamine hepatitis,[8] and antigens prepared from tissues of patients with these illnesses, but no procedure of practical value has yet been developed.

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B. Laboratory Diagnosis

The virus should be isolated and identified. Conjunctival scrapings from the patient are emulsified in veal broth and inoculated by the usual procedures into adult mice intracerebrally, into baby mice intraperitoneally and intracerebrally, and into rabbits intracerebrally and onto the scarified cornea. The animals inoculated intracerebrally or intraperitoneally should be observed daily for signs of central nervous system involvement, and the rabbits inoculated onto the cornea should be examined with a slit lamp for the development of vesicular lesions. The recovered agent should be compared in mouse neutralization tests with known positive antisera against the viruses of St. Louis encephalitis, the agent now labeled EK, and herpes simplex. Further, the patient's acute and 3 or 4 week convalescent serum specimen should be tested for neutralizing antibody against St. Louis encephalitis virus, EK virus, herpes simplex virus, and the agent recovered from the same patient. For procedures used in isolation and identification of adenoviruses, see chapter on Primary Atypical Pneumonia, pages 267 to 270 and Table 2, page 500.

XII. MEASLES (*Morbilli, Masern, Rubeola, Rougeole*)

Measles[1] is an exanthematous disease with which nearly everyone is afflicted in childhood. The illness is characterized by a prodromal period in which there is sneezing, running nose, redness of eyes, cough, fever, and the appearance of an enanthem (Koplik's spots) in the mouth. This is followed by the typical macular or maculopapular rash which spreads over the entire body. The rash leaves a brownish staining, and branny desquamation follows. Photophobia and leucopenia are usually present at the height of the disease, and encephalomyelitis is a rare complication of the illness.

The disease is caused by a filtrable virus which can be propagated in chick embryo tissue culture,[2,3] in embryonated eggs,[4,5] and in monkeys.[6,7] Recently, Enders and Peebles[8] reported recovery of 8 viruslike agents in cultures of human or simian renal cells inoculated with blood or throat washings from 5 cases of typical measles. Multiplication of the agents in vitro was accompanied by cytopathogenic changes in the cells, consisting primarily of the formation of syncytial giant cells wherein the chromatin assumes a marginal position and is replaced centrally by an acidophilic substance of unknown nature. Patients with measles de-

velop neutralizing antibodies for at least one of the agents in the progress of their disease and, in addition, develop antibodies which fix complement in the presence of antigen derived from cultures of the measles agents.

XIII MOLLUSCUM CONTAGIOSUM

A Introduction

Molluscum contagiosum is an infectious disease of the human skin which is characterized by the formation of multiple, discrete, cutaneous epithelial nodules about 2 mm in diameter These nodules are usually pearly white in appearance and present a central dimple on the surface Almost any skin area may be attacked except the soles of the feet and the palms of the hands The molluscum tumor frequently suppurates and further spread of the infection may result by scratching the area

The disease is caused by a virus,[1 2] and man appears to be the only host.[3 6] Cells infected with virus expand greatly in size, and the cytoplasm becomes filled with an acidophilic hyaline mass, spherical or pear-shaped, about 20 to 30 μ in diameter The molluscum body is generally considered to be an inclusion body which encloses a large mass of viral elementary bodies (about 250 mμ in diameter) embedded in a gelatinous matrix[5 7]

B Laboratory Diagnosis

1 *Microscopic examination of material from the lesions* A simple method for rapid diagnosis has been described by van Rooyen and Rhodes[5] The nodule after anesthetization with cocaine or freezing with dry ice, is squeezed with a forceps, and the milky exudate is collected on a glass slide One drop of either Lugol's iodine solution or 1 2,000 to 1 25,000 solution of brilliant cresyl blue in physiologic saline solution is added to the tissue, a cover slip applied, and the preparation is examined with an ordinary microscope, using the dry, high-power objective The molluscum body treated with Lugol's solution appears as a large, oval, deeply stained brown mass and, with brilliant cresyl blue, as a large blue mass inside the infected epithelial cell

2 *Complement fixation test* Mitchell[6] demonstrated complement-fixing antibody in a titer of 1 4 or 1 8 in 3 of 14 patients with molluscum contagiosum The tests were performed by the usual procedure, using soluble viral antigen obtained from molluscum contagiosum nodules, guinea pig complement, and the patients' serum

XIV. MONONUCLEOSIS, INFECTIOUS (Glandular Fever, Drüsenfieber, Monocytic Angina, Acute Benign Lymphoblastosis, Acute Mononucleosis)

A. Introduction

Infectious mononucleosis is *characterized principally by hyperplasia of the* lymphatic system, by mononucleosis, and by increase in amount of sheep cell heterophile agglutinin in the patient's serum. The disease is protean[1-4] and may present findings of fever, chills, pharyngitis with or without membrane, conjunctivitis, lymph node *enlargement (especially the cervical nodes), splenomegaly*, hepatitis, nephritis, and cutaneous rash. Involvement of the central nervous system with meningitis, encephalitis, neuronitis, or meningoencephalitis may occur in rare instances. The incidence of the disease is highest in children and young adults. The illness *is rarely fatal, and subclinical infections have been reported.*[3,5]

demonstration of significant levels of heterophile antibody may assist materially in establishing the diagnosis.

B. Blood Picture

The total white blood count varies considerably in infectious mononucleosis, but there is a general pattern which follows the progress of

the total white count may range from 10,000 to 80,000 cells. Fifty to 99 per cent of these cells are mononuclear abnormal lymphocytes and are often large, showing a foamy or deeply basophilic cytoplasm and fenestrated nuclei.[7]

C. Serodiagnosis

Between 50 and 90 per cent of patients with infectious mononucleosis develop agglutinins for sheep red blood cells during the course of their illness.[1,3,6] Such agglutinins may be present also in the sera from patients with serum sickness after injection of horse serum[8] and *in sera from* certain normal individuals.[9] The heterophile antibody present in cases of infectious mononucleosis differs from that found either in individuals with serum sickness or in normal persons and can be differentiated readily by absorption of the serum with guinea pig kidney tissue (containing Forssman antigen) and beef erythrocytes.[10] Sheep cell agglutinins of infectious mononucleosis are absorbed by beef erythrocytes but not by guinea pig kidney; the agglutinins of serum sickness are *absorbed by*

both guinea pig kidney and beef red blood cells; the agglutinins of normal serum are almost completely absorbed by guinea pig kidney but are not absorbed by beef erythrocytes.

Standard methods for performing the original Paul-Bunnell heterophile agglutination test[11] and for absorption of sera have been described by Stuart[10] These are reproduced below in slightly modified form

1 *Standard heterophile agglutination test.* To 0 5 ml volumes of *serial 2-fold dilutions of patient's serum (inactivated at 56° C. for 30 minutes)* in physiologic saline solution are added 0.5 ml. amounts of 1 per cent sheep red blood cell suspension For control, 0 5 ml of cell suspension is added to 0 5 ml of physiologic saline solution The tests are read following incubation at 37° C. for 3 hours in the water bath. The titer is expressed as the highest final dilution of serum causing definite agglutination Some workers prefer to incubate the tests at 37° C for 1 or 2 hours followed by storage overnight in the refrigerator If this is done, the tests should be rewarmed to 37° C for 2 hours before final reading in order to obviate any possible effect of sheep cell cold agglutinins which may be present in the serum[12]

With the procedure described above, titers of 1 40 or less may be considered negative, titers of 1 80 to 1 160 are regarded as presumptive, and titers of 1 320 or greater are positive As in other diseases, the demonstration of a significant increase (4-fold or greater) in amount of antibody during the progress of the illness is of far greater significance than is a positive test with a single specimen

All sera which show agglutinins for sheep cells should be absorbed with guinea pig kidney tissue and beef erythrocytes and retested to determine the kind of heterophile agglutinin which is present.

2 *Absorption test to determine the type of heterophile agglutinin.*

a Absorbing reagents *

(1) Guinea pig kidney A freshly harvested kidney is ground in a mortar (or by an equally effective method) and the emulsion is rubbed through 13xxx bolting cloth or its equivalent. The strained tissue is emulsified in 15 ml of distilled water and centrifuged at 3,000 r p m. for 15 minutes. The supernatant fluid is removed and discarded, and the operation is repeated 3 or 4 times The final sediment is diluted with

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* These are available in dried form from Cappel Laboratories, Box 352, Wayne, Pa.

physiologic saline solution to give a turbidity at least twice that of a No. 10 barium sulfate standard.[13]

Guinea pig kidney reagent may be conveniently stored in dried form. To prepare dried material, the kidneys are ground in a fine meat chopper and washed with distilled water as described above. The tissue is dried by lyophilization or in an evaporating dish at 75° C., with frequent stirring to prevent caking. The dried material is finely ground in a ball mill or in a mortar, then sifted through 13xxx bolting cloth and finally stored in a dry place. For use in absorption, the tissue is rehydrated with sufficient physiologic saline solution to give at least twice the turbidity of the No 10 barium sulfate standard. Rehydration requires several minutes and should not be hastened.

(2) Beef erythrocytes. Whole beef blood is defibrinated and the packed cells, washed 3 or 4 times in physiologic saline solution, are used to absorb serum.

b. Absorption technic. To 1 ml. of the inactivated serum diluted 1:2.5 is added 1 ml. of guinea pig kidney suspension. The mixture is incubated at 37° C. for 30 minutes in the water bath, centrifuged at high speed (4,000 r p.m.) for 15 to 20 minutes, and the supernatant fluid is tested for sheep cell agglutinins as described in section XIV, C, 1 above. It is essential that the supernatant fluid be clear after centrifugation since strong turbidity may interfere with agglutination of the sheep cells. If the agglutination test is still positive, ½ volume of packed beef erythrocytes is added to the remaining absorbed serum, and the mixture is incubated at 37° C. for 30 minutes. After centrifugation, the supernatant fluid is tested for sheep cell agglutinins as before.

c. Interpretation of test results. The sheep cell agglutinins for infectious mononucleosis are removed by absorption with beef erythrocytes but not with guinea pig kidney

3. *Rapid tests for heterophile antibody.*

a. Evans[14] described a rapid micro method for detecting heterophile antibody, using capillary blood and a white cell diluting pipette. The patient's whole blood is employed, and heat inactivation is omitted. Absorption tests are not included.

b Tannen[15] and Glassy[16] recently showed that heterophile agglutination tests performed on glass slides may be of value for rapid screening of sera before testing by the conventional method.

c. Mason[17] recently reported the development of an ox cell hemo-

lysin technic which promises to simplify greatly the test for heterophile antibody in infectious mononucleosis By this method, serial dilutions of the patient's inactivated serum are tested with standard amounts of guinea pig complement and ox cell suspension, and a hemolysis end point is employed. In tests performed with 60 sera from patients with infectious mononucleosis and 200 controls, the ox cell hemolysin technic was shown to be of equal or greater sensitivity than the sheep cell agglutination method Serum absorption is unnecessary and, in addition, valid results may be obtained in tests with a single serum dilution (1 6) since normal human sera are essentially devoid of ox cell antibody.

XV NEWCASTLE DISEASE

A INTRODUCTION

Newcastle disease (avian pneumoencephalitis, avian pseudoplague) is a highly infectious and lethal disease of fowl (chickens, turkeys, pheasants, guinea fowl, sparrows, crows, and parrots) which is caused by a virus and which affects the respiratory, gastrointestinal, and central nervous systems The malady is occasionally transmitted to human beings who either handle infected birds (poultry house workers) or who work with the virus in the laboratory [1-4] The illness in man is manifest as an acute, granular conjunctivitis (sometimes hemorrhagic) without corneal involvement Preauricular lymphadenitis, headache, malaise, and chills may also be present The disease in man is self-limited, and spontaneous recovery occurs within 2 weeks

The virus of Newcastle disease (NDV) is of spherical, filamentous, or spermlike form and is of medium size (filaments about 70 mμ by 180 mμ) [5,6] The agent is stable on storage at −70° C or in dried form.

NDV virus multiplies readily in the allantoic cavity of embryonated eggs, yielding fluids which cause hemagglutination of red blood cells from many avian and mammalian species [7] After incubation at 20° C to 37° C for prolonged periods, the virus is eluted from the cells in the same manner as mumps and influenza viruses Hemagglutination is inhibited by specific antibody to the virus and is one of the more commonly used methods for quantitating antibody in NDV antiserum. Specific antibody may also be measured by the complement fixation and *in ovo* serum neutralization technics

B. LABORATORY DIAGNOSIS

Diagnosis of NDV infection in man may be accomplished either by the isolation and identification of the causal agent or by demonstrating a significant increase (4-fold or greater) in amount of specific antibody in the patient's serum during convalescence

1. *Virus isolation* Exudate from the infected eye is collected with a sterile capillary pipette or a cotton swab and emulsified in a small volume

of sterile veal infusion or nutrient broth. If exudate is not present, saline washings of the conjunctivae may be used. The specimen is treated to contain 1,000 units each of penicillin and streptomycin per ml. and is inoculated in 0 2 ml. amount into the allantoic cavity of 9- to 11-day embryonated eggs. (Penicillin G concentrations greater than 5,000 units per ml. may have a destructive effect upon NDV virus).[6] Viral infection results in embryo death and in the appearance of hemagglutinins in the allantoic fluids 48 to 96 hours (occasionally as long as 7 days) after inoculation. Second passage should be carried out if the findings are negative on primary passage. Isolated agents may be identified by the hemagglutination-inhibition, complement fixation, or serum neutralization procedures referred to below.

For purpose of virus isolation, due care must be taken to exclude the use of embryonated eggs either from flocks which are currently or have recently experienced natural NDV infection or from flocks which have been vaccinated with the agent during the previous 30-day period. Further, positive isolation results, to be significant, should be supported by the demonstration of a 4-fold or greater increase in specific antibody in the patient's serum during convalescence from the illness.

2 *Serodiagnosis* The methodology for serodiagnosis of Newcastle disease infection in man has been neither fully developed nor evaluated. This is the natural consequence of the apparent infrequency of human infection with this agent and the resultant lack of material for study. *Investigations along these lines have been complicated* further by the existence of heat-labile and heat-stable nonspecific substances in human and animal sera which vary in amount and give false positive findings in in vivo and in vitro serologic tests [9-15] Further, marked increase in quantity of heat-stable substance capable of fixing complement with NDV antigen or of inhibiting NDV hemagglutinin may occur during convalescence from mumps virus infection.[10,14 16]

The view has been expressed[16,cf 10] that mumps and Newcastle disease viruses may be related antigenically, and this may be responsible for the positive serologic findings for NDV in mumps cases. This may not be the case since Wenner *et al.*[14,15] have reported removal of the heat-stable NDV-reactive factor from mumps serum by chemical and enzymatic methods without significant destruction of mumps antibody. It seems probable that the reaction between mumps serum and NDV virus is due entirely to nonspecific factors.

It is evident that further work will be required to elucidate and eliminate all nonspecific reactions that may occur in the NDV tests. In the meantime, a positive serologic result for NDV must be interpreted with caution, and concomitant mumps infection should always be considered Wherever possible, virus isolation studies should also be performed

The serologic technics employed by the various workers to measure NDV antibody are extremely diverse and are widely scattered in the literature It appeared worth while to incorporate the essential information into a set of suggested serologic procedures which are presented below These are intended to serve only as a guide until the eventual development of standard tests of proved reliability

a Preparation of antigen, seed virus, and control antisera for use in the serologic tests. Nine- to 11-day embryonated eggs are injected allantoically with 0.2 ml of appropriately diluted (usually 10^{-3}) infected allantoic fluid prepared from a known strain of NDV virus (a virulent strain of virus should be used since attenuated vaccine strains may be relatively nonlethal for chick embryos and may fail to cause hemagglutination under certain conditions [17] The eggs are incubated further at 35° C to 36° C until dead (2 to 3 days), and the allantoic fluids are removed after chilling at 4° C Such fluids should exhibit hemagglutinin titers of 1 320 to 1,2,560, complement fixation titers of 1.2 to 1 16 and should infect eggs in a dilution of 10^{-7} to 10^{-9} These materials are preserved in sealed glass ampules at —70° C or are lyophilized Positive control serum for use in the serologic tests may be obtained from chickens convalescent from the natural disease or from birds which have been vaccinated with an avirulent strain [18] Rabbit antisera may be prepared by injecting the adult animals intravenously or intraperitoneally with 10 0 ml amounts of heavily infected allantoic fluid on 2 occasions, 1 week apart. The immune serum is collected 2 weeks after the last injection of virus

b Handling of serum specimens All sera should be heated at 56° C. for 30 minutes prior to test In order to obviate differences in serum titer occasioned by normal variation in the test procedure from day to day, both acute and convalescent specimens from the patient *must* be tested simultaneously.

c. Hemagglutination-inhibition test. The same general procedures used for performance of the hemagglutination-inhibition test for influenza

are applicable here.[8,9,10,14 18,19,20] Human "O" or chicken erythrocytes function satisfactorily in the tests. Some workers prefer to incubate tests with chicken cells at 4° C. rather than at room temperature.[10]

A convenient hemagglutination-inhibition technic has been developed by the Committee on Standard Serological Procedures in Influenza Studies.[21] By this method, serial, 2-fold dilutions of patient's serum (1:10 to 1:2,560) in 0.25 ml. volume are allowed to react at room temperature (20° C. to 24° C.) with 4 hemagglutinating units (0.25 ml.) of antigen and 0.5 ml. of a 0.5 per cent suspension of human "O" or chicken erythrocytes. Serum titers are expressed in terms of initial dilution of serum (prior to adding the other reagents), and complete inhibition of hemagglutination is the end point. Known positive and negative control sera should always be included in the tests.

d. Complement fixation test. Several different complement fixation procedures have been used.[10,14 18,19] A convenient technic is described on pages 540-41, "Serologic Procedures for the Diagnosis of Rickettsial Diseases." By this method, serial dilutions (1:4 to 1:512) of the inactivated (56° C. for 30 minutes) serum in 0.25 ml. volume are incubated at 4° C. overnight (14 to 18 hours) with 2 exact units (0.5 ml.) of guinea pig complement and 4 units (0.25 ml.) of NDV antigen. The hemolytic system (0.5 ml. of a 1.5 per cent sheep erythrocyte suspension containing 3 units of rabbit antisheep hemolysin) is added, and the tests are read after 30 minutes' incubation in the water bath at 37° C. The serum titer is the highest initial dilution of serum causing a 3 + or greater fixation of complement. The complement titration should be carried out in the presence of 4 units of the NDV antigen. All sera should be tested with normal allantoic fluid antigen as well as with the NDV reagent, and all tests should be controlled with known positive and negative antisera. Chicken antiserum may not be used for this purpose since immune serum from this species does not fix complement in the presence of antigen. If rabbit antiserum is employed, it may be necessary to attempt removal of antiegg antibody by absorption with normal chorioallantoic membrane suspension.

e. Serum neutralization test. These tests are performed in embryonated eggs using the same general procedures as for influenza virus (see chapter on "Influenza," pp. 241-61). Serial 2-fold dilutions of serum (1:2 through 1:256) may be tested with a constant amount of virus (about 1,000 lethal doses) or serial 10-fold dilutions of virus (containing 10, 100, 1,000, 10,000, and 100,000 lethal doses of virus) may be assayed with undiluted serum.[8,9-13] Some workers have included serial dilutions

both of virus and of antiserum in the same test. Equal volumes of inactivated serum and virus are mixed and incubated at 37° C. for 30 to 60 minutes, and 0 2 ml, amounts of the mixtures are injected allantoically into 9- to 11-day embryonated eggs. The end point may be based on embryo death or upon the presence or absence of hemagglutinin in the allantoic fluid after 48 hours of incubation.

f Interpretation of serologic test results. The demonstration of a 4-fold or greater increase during convalescence of *specific* NDV antibody by the hemagglutination inhibition, complement fixation, or serum neutralization (serum dilution method) technic may be considered significant.

dose [illegible]

least [illegible]

a positive result. The laboratory results should be interpreted and evaluated in the light of the clinical findings

XVI RIFT VALLEY FEVER (Enzootic Hepatitis)

A. Introduction

Rift Valley fever is an acute febrile disease of viral etiology which affects primarily sheep and cattle[1,2] The natural disease has been reported only from Africa Human beings become infected through exposure to the virus in the laboratory[3a b] or through contact with infected animals in nature The virus has been recovered from wild-caught mosquitoes, and mosquito transmission to laboratory animals has been accomplished experimentally[4,5] It is believed that these insects may be of importance in the natural transmission of the disease

Rift Valley fever in man is generally a mild disease resembling epidemic influenza *There is a sudden onset 5 or 6 days after exposure to the agent, fever of several* days' duration, intense prostration, marked aching in the joints and extremities, abdominal discomfort, and leucopenia The eyes may be affected in certain cases with resultant blurring, retinal hemorrhage, and scotoma giving almost complete blindness[6] The temperature curve is usually of saddle-back form resembling that of dengue or yellow fever and the period of convalescence is short Serologic surveys have revealed widespread occurrence of antibody to the agent among the populations of East and Central Africa recently, and a widespread epidemic and epizootic of Rift Valley fever occurred in South Africa[6]

The virus is pathogenic for monkeys, sheep, cattle, goats, ferrets, hamsters, white rats, mice, and certain other rodents and can be grown in chick embryo tissue culture and in the embryonated egg. Horses, guinea pigs, chickens, canaries, and pigeons are insusceptible to the disease Infected animals develop discrete or coalescent focal necrosis of the liver with pinpoint hemorrhage surrounding such foci. The cytoplasm of the cells shows a hyaline degeneration, and acidophilic *intranuclear inclusion bodies may be observed.* The virus may become "fixed" during passage in mice by the cerebral route losing its viscerotropism. Ferrets inoculated intranasally develop extensive, fatal pneumonia and widespread vascular hemorrhages.[3a]

The virus of Rift Valley fever has been one of the notorious agents as regards infection of laboratory workers It is extremely hardy and will survive in infected serum for months at 4° C [3a] While vaccines are available for veterinary use, none has been developed for man Hence studies on this agent should be undertaken only after due consideration and after proper precautions have been taken to prevent its introduction into neighboring human and animal populations.

B. Laboratory Diagnosis

Laboratory diagnosis consists either of the recovery and identification of the causal agent or demonstration of significant increase in antibody for the virus in the patient's serum during convalescence from the disease.

1. *Virus isolation* The virus may be recovered from the patient's blood during the first 3 days of illness by intracerebral, intraperitoneal, or intranasal inoculation into mice. It may also be recovered readily from throat washings.[3a] The infected mice succumb within 2 or 3 days after inoculation, and the agent may be transmitted serially in mice by all routes of inoculation The recovered virus is identified in the neutralization test, using known positive antisera. Further, extensive liver necrosis with the presence of acidophilic intranuclear inclusions may be demonstrated in infected mice

2. *Serodiagnosis.*

a Serum neutralization test. Blood specimens are collected from the patient at onset of illness and again 10 days to 3 weeks later. Samples of the undiluted sera are heated to 56° C for 30 minutes to destroy the infectivity of any live virus present in the sera; these are then mixed with equal volumes of serial 10-fold dilutions of virus (suspension of livers of mice infected with a viscerotropic strain of virus), incubated at 37° C. for 30 minutes and inoculated intraperitoneally into 12 to 14 gm white mice. The mice are observed daily for illness and death, and a positive test is one in which the convalescent serum specimen neutralizes at least 100 LD_{50} more virus than does the acute phase serum.

b. Complement fixation test. Broom and Findlay[7] demonstrated complement-fixing antibodies in the sera of human beings, monkeys, sheep, rats, and mice recovered from Rift Valley fever infection. The antigen used was prepared from livers of infected mice or rats. Haig and Kaschula (quoted in 1) were unable to detect complement-fixing antibodies in sera from animals or patients infected with a laboratory strain of Rift Valley fever using antigen prepared from a virulent heterologous strain.

XVII RUBELLA (German Measles, Rubeola, Epidemic Roseola, Roteln)

This is a mild exanthematous affliction of childhood [1] There may be a short prodromal period with mild catarrhal symptoms and malaise but no fever This is followed by the development of a rash which starts on the face and neck and then extends to the trunk. The rash consists of round, pink, slightly raised macules which may be discrete or confluent, it is rarely papular and lasts for only 2 or 3 days Slight branny desquamation is sometimes seen Koplik's spots are absent, but a fine red enanthem may occur on the soft palate, the tonsils may be enlarged, and well-marked cervical and occipital lymphadenopathy is characteristic.

The disease is of viral etiology,[2] and successful passage of the agent to rhesus monkeys[3] and cultivation on the chorioallantois of the embryonated egg[3] have been reported Anderson,[4] however, was unable to demonstrate multiplication of the virus in the chick embryo

XVIII. SALIVARY GLAND VIRUS INFECTION, GENERALIZED (Inclusion Disease, Cytomegalic Inclusion Disease)

A Introduction

Generalized salivary gland virus infection is an acute and often fatal illness observed primarily in young children (for review see Reference 1) Intrauterine infection may also occur resulting in death *in utero* or during the neonatal period The clinical picture is of diverse form depending upon the distribution and severity of infection in the organs Blood dyscrasia and hepatic damage are most commonly observed among infants less than 2 months of age In children more than 2 months old, the illness is often associated with another primary disease with the salivary gland virus infection playing only a minor role There is evidence that disturbance of the cellular metabolism such as occurs in vitamin deficiency may predispose to this disease [1]

The generalized salivary gland virus infection is caused by an agent or group of agents which are widespread among mammalian species and which are species-specific [2] Experimental transmission of the infection by a viral agent has been accomplished in the rodent disease Because of the striking similarity of the inclusions seen in the infected human and animal tissues, it is commonly assumed that the salivary gland agent of man is also a virus

B Laboratory Diagnosis

Laboratory diagnosis of salivary gland virus infection is accomplished by demonstrating the characteristic inclusions in sections of tissue from organs obtained at autopsy. The liver, kidneys, pancreas, salivary gland,

lungs, adrenal gland, and intestines should be examined. The cytoplasm and nuclei of infected cells are greatly enlarged, and the nuclei contain acidophilic or purplish inclusions surrounded by a clear halo. The cell cytoplasm is acidophilic or amphophilic and may contain several small basophilic bodies measuring 2 to 4 μ in diameter. The inclusions are usually seen in epithelial cells but may occur also in mesenchymal cells.

XIX. VARICELLA (Chicken Pox) and HERPES ZOSTER (Shingles, Zona, Zoster)

A. Introduction

Varicella is a mild febrile disease of infectious nature which is accompanied by an itching vesicular eruption of the skin and mucous membranes. The individual lesions are surrounded by an area of erythema. Herpes zoster is a severely painful malady characterized by inflammation of the dorsal-root ganglia or extramedullary ganglia of cranial nerves. Vesicles identical to those of varicella are present in areas supplied by the affected sensory nerves. Varicella occurs primarily in children and may be present in epidemic form; zoster is rarely seen in persons less than 20 years old and is of sporadic occurrence.

Varicella and herpes zoster are caused by similar if not identical viruses (for discussion see Reference 1). The elementary bodies of these viruses are usually brick-shaped and are about 210 mμ by 250 mμ in size.[2,3] The nuclei of infected cells contain spheroidal acidophilic inclusion bodies which may also be found in the cell cytoplasm after nuclear degeneration.

B. Laboratory Diagnosis

There is no specific diagnostic procedure currently available. The diagnostic problem is primarily one of eliminating the presence of the virus of herpes simplex and those of the variola-vaccinia group; this can be accomplished by methods discussed elsewhere in this manual. Nagler and Rake[2,3] pointed out that the elementary bodies of varicella are smaller than those of variola and vaccinia and that observation of electron micrographs of elementary bodies purified from skin lesions of patients may be of assistance in differentiating these diseases.

Early attempts to propagate the agents of varicella and herpes zoster were unsuccessful. However, Rivers[4] observed focal lesions with intranuclear inclusions in monkey testicle after local injection of varicella vesicle fluid, and Goodpasture and Anderson[5] and Blank, Coriell, and Scott[6] demonstrated intranuclear inclusions in human skin grafted onto the chorioallantoic membrane of embryonated eggs and inoculated with zoster material.

Sprunt and Hirst[7] reported isolation of several new rickettsia-like

agents from chickenpox vesicle fluid. These agents produced pocks on the chorioallantoic membrane, multiplied rapidly in the yolk sac of embryonated eggs and were pathogenic for guinea pigs by several routes of inoculation The origin of these rickettsiae as well as their relationship to human or other naturally occurring disease was obscure. More recently, Weller[8] reported serial propagation of a number of strains of a cytopathogenic agent in the roller tube cultures of human skin, embryonic skin-muscle, or child's (3 months to 3 years) foreskin inoculated with vesicle fluid from patients with varicella or zoster Patients with varicella showed an increase in complement-fixing antibody for antigen prepared from varicella passage material, but the specificity of the reaction remained to be proved; neutralizing antibody could not be demonstrated

XX. WARTS

Human warts may appear in several different forms which are called common, juvenile (plane), filiform, digitate, or genital warts and laryngeal papillomata Warts are of viral etiology,[1,3] and it is believed that all the different types are caused by a single virus.[4] Successful propagation of human wart virus in canine vaginal mucosa and rabbit testicle has been reported, but other attempts to grow the agent in laboratory animals have not been successful (for discussion see References 4 and 5) More recently, Bivins[5] employed methods used for commercial manufacture of animal wart vaccine and recovered an agent from human wart tissue which produced whitish "pearl-like" lesions on the chorioallantoic membrane of the embryonated egg Unfortunately, the identity of the agent was not established Acidophilic intranuclear inclusions and "cytoplasmic masses" have been observed in ordinary microscopic sections of wart tumor tissue and "virus-like" particles have been seen by means of the electron microscope[6,7,cf 8]

Suitable laboratory methods for diagnosis of warts are not now available, but the disease is sufficiently pathognomonic to obviate laboratory confirmation of the clinical diagnosis.

TABLE 2

SUMMARY OF DIAGNOSTIC TESTS FOR MISCELLANEOUS VIRAL DISEASES

Disease	Virus Isolation Tests: Animal of choice	Virus Isolation Tests: Specimen from patient	Virus Isolation Tests: Inoculation route	Virus Isolation Tests: Illness in animal	Virus Isolation Tests: Inclusions	In Vivo Immunologic Tests: Test	In Vivo Immunologic Tests: Common virus source	In Vivo Immunologic Tests: Usual test animal	In Vivo Immunologic Tests: Inoculation route	In Vitro Serologic Tests: Test	In Vitro Serologic Tests: Common antigen source
I. Anemia, equine infectious	Horse	Serum	Subcutaneous	Fever, anemia, edema, emaciation							
II. Cat scratch disease						Skin test in patient	Human pus or lymph node			Complement fixation for LGV on experimental basis	LGV infected egg (yolk sac)
IV Colorado tick fever	Suckling mouse	Acute phase whole blood or serum	I.C. and I.P.	Central nervous system disease		Serum neutralization	Infected mouse brain	Mouse	I.C.	Complement fixation	Infected mouse brain
V Ecthyma, contagious	Lamb	Pus, emulsified scab	Scarification	Pustular lesions						Complement fixation	Vesicle fluid from infected sheep
VI. Encephalomyocarditis	Mouse	Spinal fluid, brain, cord, myocardium, spleen	I.C.	Central nervous system disease		Serum neutralization	Infected mouse brain	Mouse	I.C.	Complement fixation	Infected mouse brain
VII. Exanthem subitum (experimental)	Human cell tissue culture including strain Hela	Stool throat washings		Cytopathogenic change. Look for a virus of the adeno group		Serum neutralization	Infected Hela cell culture	Hela cell tissue culture		Complement fixation	Hela cell culture infected with a virus of the adeno group
VIII. Foot-and-mouth disease	Guinea pig	Vesicle fluid	Foot pad	Vesicular lesions		Serum neutralization	Infected guinea pig, vesicle fluid	Suckling mouse	I.C.	Complement fixation	Infected suckling mouse brain, bovine tongue epithelium tissue culture
	Suckling mouse	Vesicle fluid	Intracerebral	Spastic paralysis-death			Suckling mouse brain	Guinea pig	Foot pad		
XI. Keratoconjunctivitis, epidemic	Adult and baby mice	Conjunctival scrapings	I.C. and I.P.	Central nervous system disease	Acidophilic intranuclear	Serum neutralization	Infected mouse or rabbit brain	Mouse	I.C.		
	Rabbits (experimental)	Conjunctival scrapings	I.C. and onto cornea	Vesicular lesions on rabbit cornea	Acidophilic intranuclear						
	Hela cell tissue culture	Conjunctival washings		Cytopathogenic change. Look for a virus of the adeno group		Serum neutralization	Infected Hela cell culture	Hela cell tissue culture		Complement fixation	Hela cell culture infected with a virus of the adeno group

XII. Measles	Human or simian renal cells in tissue culture	Throat washings, blood		Cytopathogenic effect. Syncytial *giant cells* with margination of chromatin and central replacement in the nucleus with an acidophilic substance	Acidophilic substance *centrally in* nucleus	Serum neutralization	Infected renal tissue culture	Human or simian renal tissue culture		Complement fixation	Infected renal tissue culture
XIII. Molluscum contagiosum		Exudate from lesion examined for molluscum bodies			Molluscum bodies					Complement fixation	Human lesions
XIV Mononucleosis, infectious										Heterophile agglutinin Hemolysin for beef cells	
XV Newcastle disease	Embryonated egg	Ocular exudate or conjunctival washings	Allantoic	Death (hemagglutinins in allantoic fluid)		Serum neutralization	Infected allantoic fluid from embryonated egg	Embryonated egg	Allantoic	Complement fixation H I	Infected allantoic fluid-egg Infected allantoic fluid-egg
XVI. Rift Valley fever	Mouse	Acute phase blood. Throat washings	I.C and I P	Hepatic necrosis	Acidophilic intranuclear	Serum neutralization	Infected mouse liver	Mouse	I P	Complement fixation	Infected mouse liver
XVIII. Salivary gland virus infection, generalized		Liver, kidney, pancreas, salivary gland, lungs, adrenal gland, intestines examined histologically			Basophilic cytoplasmic						
XIX. Varicella Herpes zoster	Human skin tissue culture	Vesicle fluid		Cytopathogenic effect							
XX. Warts		Wart lesion examined histologically									

Note No suitable laboratory tests are available for

III Cold, common, IX Gastroenteritis, epidemic viral, X. Hepatitis, viral, A and B, XVII Rubella

Abbreviations. I C = Intracerebral I.P = Intraperitoneal H.I. = Hemagglutination-Inhibition

XXI. REFERENCES

I. Anemia, Equine Infectious

1. Warren, J Infections of Minor Importance, Chapter 39 in *Viral and Rickettsial Infections of Man*, 2d ed. Edited by T. M Rivers, Philadelphia: J. B. Lippincott Company, 1952, pp 668–669.
2. Morris, J A., and Alexander, A D Attempts to Adapt Equine Infectious Anemia Virus to Small Animals. *Cornell Vet.*, 40 381–383, 1950.
3. Giltner, L T. Personal communication.

II. Cat Scratch Disease

1. [illegible]
2. [illegible]
3. Blattner, R [illegible]
4. Mollaret, P [illegible] nouvelle. [illegible] réaction et [illegible]
5. Mollaret, P [illegible] l'adénopathie regionale subaiguë et spontanément curable decrite en 1950, la lymphoréticulose bénigne d'inoculation. *Presse Méd*, 58 1353–1355, 1950.
6. Greer, W E. R, and Keefer, C S. Cat Scratch Fever; a Disease Entity. *New England J Med.*, 244 545–548, 1951.
7. Siguier, F., Welti, J. J., and Lumbroso, P. Nouvelles observations de formes pseudo-vénériennes de la maladie des griffes du chat *Bull. et Mém. Soc. Méd Hôp. Paris*, 68 216–219, 1952.
8. Anonymous. Cat Scratch Disease. *J. A. M. A.*, 148 746–747, 1952.
9. Thompson, T. E., Jr., and Miller, K. F Cat Scratch Encephalitis. *Ann. Int. Med.*, 39:146–151, 1953
10. Anonymous. Cat Scratch Disease and Parinaud's Oculoglandular Syndrome *J. A. M. A.*, 152 1717, 1953.
11. Mollaret, P., Reilly, J, Bastin, R, and Tournier, P. La Découverte du virus de la [illegible]
12. [illegible] colorations. *Presse Méd*, 59 701–704, 1951.
13. Nigg, C, Hilleman, M R, and Bowser, B M Studies on Lymphogranuloma Venereum Complement-Fixing Antigens. I. Enhancement by Phenol or Boiling. *J. Immunol*, 53 259–268, 1946

III. Cold, Common

1. Kruse, W. Die Erreger von Husten und Schnupfen *München Med. Wchnschr.*, 61: 1547, 1914
2. Foster, G. B, Jr. The Etiology of Common Colds The Probable Role of a Filterable Virus as the Causative Factor. a Preliminary Note. *J. A. M. A*, 66 1180–1183, 1916
3. [illegible]
4. [illegible] mon Cold and Its Cultivation in Tissue Medium *Proc. Soc. Exper. Biol. and Med*, 28 513–516, 1931.
5. Kneeland, Y, Jr, Mills, K C., and Dochez, A. R. Cultivation of the Virus of the Common Cold in the Chorioallantoic Membrane of the Chick Embryo. *Proc. Soc Exper. Biol. and Med*, 35 213–215, 1936.

6 Pollard, M, and Caplovitz, C D Experimental Studies with the Agent of the Common Cold *Science*, 106 243–244, 1947
7 Topping, N H, and Atlas, L T The Common Cold A Note Regarding Isolation of an Agent *Science*, 106 636–637, 1947
8 Ward, T G, and Proctor, D F Isolation of a Common Cold Virus in Chick Embryos and the Clinical Manifestations It Produces in Human Volunteers *Am J Hyg.*, 52 91–106, 1950
9 Andrewes, C H, Chapromere, D M, Gompels, A E H, Pereira, H G, and Roden, A T Propagation of Common Cold Virus in Tissue Cultures *Lancet*, 2 546–547 1953
10
11.
12
61 197–218, 1955
13 Ginsberg, H S, Gold, E, Jordan, W S, Jr, Katz, S, Badger, G F, and Dingle, J Relation of the New Respiratory Agents to Acute Respiratory Diseases. *Am J Pub Health*, 45 915–922, 1955

IV. Colorado Tick Fever

1.
2
3.
mission of the Virus in the Tick *J Immunol*, 64 257–263, 1950
4.
5
6.
Adapted Virus *J Immunol*, 57 255–262, 1947
7. Koprowski, H, and Cox, H R. Colorado Tick Fever I Studies on Mouse Brain Adapted Virus *J Immunol*, 57 239–253, 1947
8. DeBoer, C J, Kunz, H J, Koprowski, H, and Cox, H R. Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever *Proc. Soc Exper. Biol. and Med*, 64 202–208, 1947
9. Cox, H R. Specific Complement-Fixing Diagnostic Antigens for Viral and Rickettsial Diseases. *Am J. Pub Health*, 38 351–360, 1948.

V. Ecthyma, Contagious

1. ata Concerning Its *Dermat and Syph.*,
2. Infection of Man *al J. Sc.*, 9.73–74, 1946.
3.
4
5
6. Giltner, L. T. Personal communication.

XXI. REFERENCES

I. Anemia, Equine Infectious

1. Warren, J. Infections of Minor Importance, Chapter 39 in *Viral and Rickettsial Infections of Man*, 2d ed. Edited by T. M. Rivers, Philadelphia: J. B. Lippincott Company, 1952, pp. 653–669.
2. Morris, J. A., and Alexander, A. D. Attempts to Adapt Equine Infectious Anemia Virus to Small Animals. *Cornell Vet.*, 40:351–383, 1950.
3. Gilmer, L. T. Personal communication.

II. Cat Scratch Disease

1. Daniels, W. B., and MacMurray, F. G. Cat Scratch Disease; Nonbacterial Regional Lymphadenitis. A Report of 60 Cases. *Ann. Int. Med.*, 37:679–713, 1952.
2. Debré, R., Lamy, M., Jammet, M. L., Costil, L., and Mozziconacci, P. La Maladie des griffes du chat. *Bull. et Mém. Soc. Méd. Hôp. Paris*, 66:76–79, 1952.
3. Blattner, R. J. Cat-Scratch Fever. *J. Pediat.*, 39:123–124, 1951.
4. Mollaret, P., Reilly, J., Bastin, R., and Tournier, P. Une maladie ganglionnaire nouvelle. Adénopathie régionale subaiguë spontanément curable avec intradermo-réaction et lésions histologiques particulières. *Presse Méd.*, 58:282–283, 1950.
5. Mollaret, P., Reilly, J., Bastin, R., and Tournier, P. Documentation nouvelle sur l'adénopathie régionale subaiguë et spontanément curable décrite en 1950, la lymphoréticulose bénigne d'inoculation. *Presse Méd.*, 58:1353–1355, 1950.
6. Greer, W. E. R., and Keefer, C. S. Cat-Scratch Fever; a Disease Entity. *New England J. Med.*, 244:545–548, 1951.
7. Signier, F., Welti, J. J., and Lumbroso, P. Nouvelles observations de formes pseudo-vénériennes de la maladie des griffes du chat. *Bull. et Mém. Soc. Méd. Hôp. Paris*, 68:216–219, 1952.
8. Anonymous. Cat Scratch Disease. *J. A. M. A.*, 148:746–747, 1952.
9. Thompson, T. E., Jr., and Miller, K. F. Cat Scratch Encephalitis. *Ann. Int. Med.*, 39:146–151, 1953.
10. Anonymous. Cat Scratch Disease and Parinaud's Oculoglandular Syndrome. *J. A. M. A.*, 152:1717, 1953.
11. Mollaret, P., Reilly, J., Bastin, R., and Tournier, P. La Découverte du virus de la lymphoréticulose bénigne d'inoculation. I. Caractérisation sérologique et immunologique. *Presse Méd.*, 59:681–682, 1951.
12. Mollaret, P., Reilly, J., Bastin, R., and Tournier, P. La Découverte du virus de la lymphoréticulose bénigne d'inoculation. II. Inoculation expérimentale au singe et colorations. *Presse Méd.*, 59:701–704, 1951.
13. Nigg, C., Hilleman, M. R., and Bowser, B. M. Studies on Lymphogranuloma Venereum Complement-Fixing Antigens. I. Enhancement by Phenol or Boiling. *J. Immunol.*, 53:259–268, 1946.

III. Cold, Common

1. Kruse, W. Die Erreger von Husten und Schnupfen. *München. Med. Wchnschr.*, 61:1547, 1914.
2. Foster, G. B., Jr. The Etiology of Common Colds. The Probable Role of a Filterable Virus as the Causative Factor: a Preliminary Note. *J. A. M. A.*, 66:1180–1183, 1916.
3. [illegible]
4. [illegible]
5. [illegible] Common Cold [illegible] *Exper. Biol. and Med.*, 35:213–215, 1936.

6 Pollard, M., and Caplovitz, C D Experimental Studies with the Agent of the Common Cold *Science*, 106 243–244, 1947
7. Topping, N H, and Atlas, L T The Common Cold A Note Regarding Isolation of an Agent *Science*, 106 636–637, 1947
8. Ward, T G, and Proctor, D F Isolation of a Common Cold Virus in Chick Embryos and the Clinical Manifestations It Produces in Human Volunteers *Am J. Hyg.*, 52 91–106, 1950
9. Andrewes, C H, Chapromere, D M, Gompels, A E H, Pereira, H G, and Roden, A T Propagation of Common Cold Virus in Tissue Cultures *Lancet*, 2 546–547 1953.
10 Hilleman, M R, Werner, J H, Dascomb, H E, Butler, R L, and Stewart, M. T Epidemiology of RI (RI-67) Group Respiratory Virus Infections in Recruit Popu-

11

12

61 197–218, 1955
13 Ginsberg, H S, Gold, E, Jordan, W S, Jr, Katz, S, Badger, G F, and Dingle, J. Relation of the New Respiratory Agents to Acute Respiratory Diseases *Am J Pub Health*, 45 915–922, 1955

IV. Colorado Tick Fever

1 Florio, L., Mugrage, E R, and Stewart, M O Colorado Tick Fever. *Ann Int. Med*, 25 466–472, 1946
2

3.

mission of the Virus in the Tick *J Immunol*, 64 257–263, 1950
4 Florio, L, Stewart, M O, and Mugrage, E R The Experimental Transmission of Colorado Tick Fever *J Exper Med*, 80 165–188, 1944
5 Oliphant, J W, and Tibbs, R O Colorado Tick Fever Isolation of Virus Strains by Inoculation of Suckling Mice *Pub Health Rep*, 65 521–522, 1950
6. Koprowski, H, and Cox, H R Colorado Tick Fever II Studies on Chick Embryo Adapted Virus *J Immunol*, 57 255–262, 1947
7. Koprowski, H, and Cox, H R Colorado Tick Fever I Studies on Mouse Brain Adapted Virus *J Immunol*, 57 239–253, 1947
8 DeBoer, C J, Kunz, H J, Koprowski, H, and Cox, H R Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever *Proc Soc Exper Biol and Med*, 64 202–208, 1947
9 Cox, H R Specific Complement-Fixing Diagnostic Antigens for Viral and Rickettsial Diseases *Am J Pub Health*, 38 351–360, 1948

V. Ecthyma, Contagious

1 Kingery, L B, and Dahl, J Ecthyma Contagiosum in Man, Data Concerning Its Incidence in Several Western States, Report of a Case. *Arch Dermat and Syph.*, 51 359–364, 1945
2 Carne, H R., Wickham, N, Whitten, W. K, and Lockley, R. P. Infection of Man by the Virus of Contagious Pustular Dermatitis of Sheep *Austral. J. Sc*, 9 73–74,
3. Transmission
–632, 1951.
4 h the Virus of Contagious Pustular Dermatitis *Lancet*, 1 720–721, 1951
5. MacDonald, A Complement Fixation Tests in the Diagnosis of Contagious Pustular Dermatitis Infection in Man. *J. Path. and Bact.*, London, 6 758–761, 1951.
6. Giltner, L T Personal communication.

XXI. REFERENCES

I Anemia, Equine Infectious

1. Warren, J Infections of Minor Importance, Chapter 39 in *Viral and Rickettsial Infections of Man*, 2d ed Edited by T. M Rivers, Philadelphia: J. B. Lippincott Company, 1952, pp 668–669.
2. Morris, J A, and Alexander, A. D Attempts to Adapt Equine Infectious Anemia Virus to Small Animals. *Cornell Vet*, 40 381–383, 1950.
3. Giltner, L T. Personal communication.

II. Cat Scratch Disease

1.
2.
3.
4.
5.
6. *land J Med.*, 244 545–548, 1951.
7. Siguler, F., Welti, J J, and Lumbroso, P. Nouvelles observations de formes pseudo-vénériennes de la maladie des griffes du chat. *Bull. et Mém Soc. Méd. Hôp. Paris*, 68 216–219, 1952
8. Anonymous Cat Scratch Disease. *J A M. A*, 148 746–747, 1952.
9. Thompson, T. E, Jr., and Miller, K F Cat Scratch Encephalitis. *Ann. Int. Med.*, 39. 146–151, 1953.
10. Anonymous Cat Scratch Disease and Parinaud's Oculoglandular Syndrome *J. A M. A.*, 152 1717, 1953
11. Mollaret, P., Reilly, J, Bastin, R, and Tournier, P. La Decouverte du virus de la lymphoréticulose bénigne d'inoculation I Caractérisation sérologique et immu-
12.
13. *munol*, 53 259–268, 1946

III. Cold, Common

1. Kruse, W. Die Erreger von Husten und Schnupfen. *Munchen Med Wchnschr.*, 61 1547, 1914
2. Foster, G B, Jr. The Etiology of Common Colds The Probable Role of a Filterable Virus as the Causative Factor· a Preliminary Note *J. A. M A*, 66 1180–1183, 1916.
3.
4.
5. *Exper. Biol. and Med*, 35 213–215, 1936

8 Neva, F. A, and Enders, J F Cytopathogenic Agents Isolated from Patients During an Unusual Epidemic Exanthem *J Immunol*, 72 307–314, 1954

VIII Foot-and-Mouth Disease

1 Hagan, W A *The Infectious Diseases of Domestic Animals with Special Reference to Etiology, Diagnosis, and Biologic Therapy* 2d ed Ithaca, New York Comstock Publishing Company, Inc, 1951, pp 649–668
2. Olitsky, P K In *Brennemann's Practice of Pediatrics* Hagerstown, Md W. F. Prior Company, Inc, 1951, v 2, Chapter VII, pp 1–3
3 Skinner, H H Propagation of Strains of Foot-and-Mouth Disease Virus in Unweaned White Mice *Proc Roy Soc Med*, 44 1041–1044, 1951
4 Galloway, I A, Henderson, W M, and Brooksby, J B Strains of the Virus of Foot and-Mouth Disease Recovered from Outbreaks in Mexico *Proc Soc Exper Biol and Med*, 69 57–63, 1948
5 Public Law 496, 80th Congress, Chap 229, 2D Session, S-2038, April 21, 1948
6 Hanson, R P, Rasmussen, A F, Jr, Brandly, C A, and Brown, J W Human Infection with the Virus of Vesicular Stomatitis *J Lab and Clin Med*, 36 754–758, 1950
7
8
9
10
11
12
13
Vet Res, 12 187–190, 1951

IX Gastroenteritis, Epidemic Viral

1 Reimann, H A, Hodges, J H, and Price, A H Epidemic Diarrhea, Nausea and Vomiting of Unknown Cause *J A M A*, 127 1–6, 1945
2 Cook, G T, and Marmion, B P Gastro-Enteritis of Unknown Aetiology An Outbreak in a Maternity Unit *Brit M J*, 2 446–450, 1947
3
4.
New England J Med, 244 749–753, 1951
5 Reimann, H A, Price, A H, and Hodges, J H The Cause of Epidemic Diarrhea, Nausea and Vomiting (Viral Dysentery?) *Proc Soc Exper, Biol and Med*, 59 8–9, 1945
6 Gordon, I, Ingraham, H S, and Korns, R. F. Transmission of Epidemic Gastroenteritis to Human Volunteers by Oral Administration of Fecal Filtrates. *J Exper Med*, 86 409–422, 1947
7 Jordan, W. S, Jr, Gordon, I, and Dortance, W R. A Study of Illness in a Group of Cleveland Families VII Transmission of Acute Non-Bacterial Gastroenteritis to Volunteers Evidence for Two Different Etiologic Agents. *J Exper. Med*, 98 461–475, 1953

7. Blakemore, F, Abdussalam, M, and Goldsmith, W. N. A Case of Orf (Contagious Pustular Dermatitis). Identification of the Virus. *Brit. J. Dermat. and Syph.*, 60 404–409, 1948

VI Encephalomyocarditis

1 Warren, J Infections of Minor Importance, Chapter 39 in *Viral and Rickettsial Infections of Man*, 2d ed Edited by T. M. Rivers, Philadelphia: J. B. Lippincott Company, 1952, pp 675–678
2. Verlinde, J D, and Hofman, B Pathogenic and Immunologic Properties of Two New Members of the Columbia SK Group of Viruses. *Arch. ges. Virusforsch*, 5. 14–26, 1952.
3 Warren, J, Russ, S B, and Jeffries, H Neutralizing Antibody Against Viruses of the Encephalomyocarditis Group in the Sera of Wild Rats *Proc. Soc Exper. Biol. and Med*, 71 376–378, 1949.
4. Smadel, J. E, and Warren, J The Virus of Encephalomyocarditis and Its Apparent Causation of Disease in Man. *J Clin Investigation*, 26.1197, 1947.
5 Dick, G. W A, Best, A M, Haddow, A J, and Smithburn, K C. Mengo Encephalomyelitis, A Hitherto Unknown Virus Affecting Man *Lancet*, 2 286–289, 1948.
6 Reed, L J, and Muench, H A Simple Method of Estimating Fifty Per Cent Endpoints *Am J. Hyg*, 27 493–497, 1938
7 Warren, J, Smadel, J E, and Russ, S. B The Family Relationship of Encephalomyocarditis, Columbia-SK, M M, and Mengo Encephalomyelitis Viruses. *J. Immunol*, 62 387–398, 1949.
8 Hallauer, C. Agglutination von Hammel erythrocyten durch murine Poliomyelitisvirusstämme. *Proc 4th Internat Cong Microbiol*, 257, 1947.
9 Olitsky, P K., and Yager, R. H Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses. Experiments ... *d Med*, 71 719–724, 1949.
10 ... 'oliomyelitisvirusstamme. *Arch. ges*
11 ... Col-MM-Virus *Proc Soc. Exper.*
12 ... Influenza Virus and Antibodies by ... *Med*, 75 49–64, 1942.
13 Salk, J E A Simplified Procedure for Titrating Hemagglutinating Capacity of Influenza-Virus and the Corresponding Antibody *J. Immunol*, 49 87–98, 1944

VII Exanthem Subitum

1 Rake, G Exanthem Subitum Chapter 24 in *Viral and Rickettsial Infections of Man*, 2d ed Edited by T M Rivers Philadelphia J. B Lippincott Company, 1952, pp. 489–490
2 Kempe, C. H, Shaw, E B, Jackson, J R, and Silver, H. K Studies on the Etiology of Exanthem Subitum (Roseola Infantum) *J Pediat*, 37.561–568, 1950
3 Neva, F A, and Enders, J F Isolation of a Cytopathogenic Agent from an Infant with a Disease in Certain Respects Resembling Roseola Infantum *J Immunol*, 72 315–321, 1954
4 Rowe, W. P, Huebner, R J, Hartley, J W, Ward, T. G, and Parrott, R. H. Studies of the Adenoidal-Pharyngeal-Conjunctival (APC) Group of Viruses *Am J Hyg*, 61 197–218, 1955
5 Hilleman, M R, Werner, J H, Dascomb, H E, Butler, R L, and Stewart, M T Epidemiology of RI (RI-67) Group Respiratory Virus Infections in Recruit Populations *Am J Hyg*, 62 29–43, 1955
6 ... of RI
7. ... J H ... n, J.

9 Meyer, H. M, Jr, and Duffy, C. E Age Susceptibility Pattern of the Rat to Epidemic Keratoconjunctivitis Virus *Proc Soc Exper Biol and Med*, 82 751–754, 1953
10 Jawetz, E, Kimura, S., Thygeson, P, Coleman, V R, and Hanna, L Viruses Isolated from Patients with Epidemic Keratoconjunctivitis *Bact Proc*, p 75, 1955
11 Huebner, R. J, Bell, J A, Rowe, W P, Ward, T G, Suskind, R G, and Hartley, J W Studies of Adenoidal-Pharyngeal Conjunctival Vaccines in Volunteers *J.A.M.A*, 159 986–989, 1955
12 Hilleman, M R., Werner, J H, Dascomb, H E, Butler, R L, and Stewart, M T *Epidemiology of RI (RI 67) Group Respiratory Virus Infections in Recruit Populations* *Am J Hyg*, 62 29–43, 1955
13 Hilleman, M R, Werner, J H, and
RI (Prototype RI-67) Viruses *Proc*
14 Rowe, W. P, Huebner, R J, Hartley,
of the Adenoidal-Pharyngeal-Conjunc
15
16
Outbreak of Febrile Pharyngitis and Conjunctivitis Associated with Type 3 Adenoidal-Pharyngeal-Conjunctival Virus Infection *N E J Med*, 251 1087–1090, 1954
17 *Dascomb, H E and Hilleman, M R Clinical and Laboratory Studies in Patients with* Respiratory Disease Caused by RI Viruses *Am J Med* In press, 1956

XII MEASLES

1 Rake, G Measles Chapter 22 in *Viral and Rickettsial Infections of Man*, 2d ed Edited by T M Rivers Philadelphia J B Lippincott Company, 1952, pp 478–483
2 Plotz, H Culture "in vitro" du virus de la rougeole *Bull Acad de Méd, Paris*, 119 598–601, 1938
3 Rake, G., Shaffer, M F, and Jones, H P Studies on Measles III The Use of *Tissue Culture in Propagation of Measles Virus J Infect Dis.*, 69 65–69, 1941
4 Rake, G, and Shaffer, M F Propagation of the Agent of Measles in the Fertile Hen's Egg *Nature* 144 672–673, 1939.
5
6.
7
8 Enders, J F, and Peebles, T C Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles. *Proc. Soc Exper Biol and Med*, 86 277–286, 1954

XIII MOLLUSCUM CONTAGIOSUM

1. Ju
2 Wi
3. Fi
Office, 1930, 7 248–251.
4 *Goodpasture, E W, and Woodruff, C. E. A Comparison of the Inclusion Bodies of* Fowl pox and Molluscum Contagiosum *Am J Path*, 7.1–8, 1931.
5 Van Rooyen, C E, and Rhodes, A J. *Virus Diseases of Man*, 2d ed. New York Thomas Nelson and Sons, 1948, pp 145–149.

8 Light, J. S, and Hodes, H. L Studies on Epidemic Diarrhea of the New-Born: Isola ... Health, 33.

9 ... ses. J. Exper.

10 ... Transmissible

11 Buddingh, G J, and Dodd, K Stomatitis and Diarrhea of Infants Caused by a Hitherto Unrecognized Virus J Pediat, 25 105–113, 1944.

12 Buddingh, G. J Virus Stomatitis and Virus Diarrhea of Infants and Young Children. South M. J, 39 382–390, 1946.

13 Keitel, H G. Occurrence of Cold and Streptococcus MG Agglutinins in Infants with Gastroenteritis. J. Infect Dis., 86 219–222, 1950.

X Hepatitis, Viral, A and B

1. Havens, W. P, Jr, and Paul, J R. Infectious Hepatitis and Serum Hepatitis. Chapter XV in *Viral and Rickettsial Infections of Man*, 2d ed. Edited by T. M. Rivers. Philadelphia J B Lippincott Company, 1952, pp. 359–377.
2 Stokes, J, Jr Viral Hepatitis Am. J. M. Sc, 225.349–357, 1953.
3 Henle, W., Harris, S, Henle, G, Harris, T. N., Drake, M. E, Mangold, F., and Stokes, J, Jr Studies on the Agent of Infectious Hepatitis. I. Propagation of the Agent in Tissue Culture and in the Embryonated Hen's Egg. J. Exper. Med., 92:

4.

5

6.

7

8.

XI. Keratoconjunctivitis, Epidemic

1. Hobson, L C Acute Epidemic Superficial Punctate Keratitis. Am. J Ophth., 21. 1153–1155 1938
2. ... ivitis"). I. Isola-

3 ... I. Isolation and Identification of a Filterable Virus. J Exper. Med, 77.71–96, 1943.
4. Calkins, H E, and Bond, G C Adaptation of Virus of Epidemic Keratoconjuncti-

5

6. R

7. R ... and St Louis Encephalitis Viruses Proc Soc. Exper. Biol. and Med, 78 483–486, 1951.
8. Cheever, F. S A Possible Relationship between Viruses of St Louis Encephalitis and Epidemic Keratoconjunctivitis Proc. Soc. Exper. Biol. and Med, 77.125–129, 1951.

5. Bang, F. B Filamentous Forms of Newcastle Virus *Proc Soc Exper Biol and Med.*, 63 5–7, 1946
6. Cunha, R., Weil, M L., Beard, D., Taylor, A R., Sharp, D G., and Beard, J W Purification and Characters of the Newcastle Disease Virus (California Strain). *J Immunol.*, 55 69–89, 1947
7 Clark, E., and Nagler, F P O Haem-agglutination by Viruses, The Range of Susceptible Cells with Special Reference to Agglutination by Vaccinia Virus. *Australian J Exper, Biol and M Sc*, 21 103–106, 1943
8 Kohn, A *In Vitro* Inactivation of Newcastle Disease Virus by Penicillin Preparations *Proc Soc Exper Biol and Med*, 82 612–616, 1953
9 Ginsberg, H S, and Horsfall, F L, Jr A Labile Component of Normal Serum Which Combines with Various Viruses Neutralization of Infectivity and Inhibition of Hemagglutination by the Component *J Exper Med*, 90 475–495, 1949
10 Kilham, L., Jungherr, E., and Luginbuhl, R E Antihemagglutinating and Neutralizing Factors Against Newcastle Disease Virus (NDV) Occurring in Sera of Patients Convalescent from Mumps *J Immunol*, 63 37–49, 1949
11 [illegible] icance of [illegible] *Hopkins* [illegible]
12. [illegible] Test for [illegible]
13 Bang, F B, Foard, M, and Karzon, D T Mode of Action of Heat Labile Serum Inactivating Substance on Newcastle Disease Virus *Bull Johns Hopkins Hosp*, 88 83–100, 1951
14 Wenner, H A, Jenson, M H, and Monley, A, A Study of Infections Caused by Mumps and Newcastle Disease Viruses I Specific and Nonspecific Serologic Relations *J Immunol*, 68 343–356, 1952
15 Wenner, H A, Monley, A, and Jenson, M H A Study of Infections Caused by Mumps and Newcastle Disease Viruses II Some Properties of Specific and Nonspecific Serum Hemagglutination Inhibitor Components *J. Immunol*, 68 357–368, 1952
16 Jordan, W S, and Feller, A E The Relationship of Complement-fixing and Antihemagglutinating Factors Against the Viruses of Mumps and Newcastle Disease *J Lab and Clin Med*, 36 369–377, 1950
17 Liu, C, and Bang, F B An Analysis of the Difference Between a Destructive and a Vaccine Strain of NDV (Newcastle Disease Virus) in the Chick Embryo *J Immunol*, 70 538–548, 1953
18 Clancy, C F, Cox, H R, and Bottorff, C A Laboratory Experiments with Living Newcastle Disease Vaccine *Poultry Sc*, 28 58–62, 1949
19 Wenner, H A, Monley, A, and Todd, R N Studies on Complement Fixation with Newcastle Disease Virus *J Immunol*, 64 323–333, 1950
20 U. S Dept. of Agriculture, Bureau of Animal Industry The Diagnosis of Newcastle Disease Mimeographed procedure dated Aug 15, 1946
21 Committee on Standard Serological Procedures in Influenza Studies An Agglutination Inhibition Test Proposed as a Standard of Reference in Influenza Diagnostic Studies *J Immunol*, 65 347–353, 1950

XVI Rift Valley Fever

1 Henning, M W Rift Valley Fever *South African Vet Med Assoc J.*, 23 65–78, 1952.
2 Theiler, M Rift Valley Fever Chapter XXXII in *Viral and Rickettsial Infections of* [illegible] tt Company,
3a [illegible] hree Cases of [illegible] the Disease to Ferrets. *J Exper Med*, 62 433–448, 1935

6 Mitchell, J C Observations on the Virus of Molluscum Contagiosum *Brit. J. Exper Path*, 34 44–49, 1953.
7 Warren, J Infections of Minor Importance. Chapter 39 in *Viral and Rickettsial Infections of Man*, 2d ed Edited by T. M. Rivers Philadelphia· J B Lippincott Company, 1952, pp. 671–672.

XIV Mononucleosis, Infectious

1 leosis at the New Haven Hospital, , 1947.
2 ctious Mononucleosis; A Study of , 1951.
3 Kimbrough, R. C., Jr. Infectious Mononucleosis or a New Entity? *J. Tennessee M A*, 44 412–418, 1951
4 Rivers, T M Infectious Mononucleosis Chapter 29 in *Viral and Rickettsial*
5.
6
7.
8. Davidsohn, I Heterophile Antibodies in Serum Sickness *J. Immunol*, 16·259–273, 1929.
9 Friedemann, U. Uber heterophile Normalamboceptoren. Ein Beitrag zur Lehre von der Entstehung der normalen Antikörper *Biochem Ztschr*, 80 333–356, 1917.
10 Stuart, C A. Infectious Mononucleosis In *Diagnostic Procedures and Reagents; Technics for the Laboratory Diagnosis and Control of the Communicable Diseases*, 3d ed. New York American Public Health Association, 1950, pp. 308–314.
11 Paul, J. R, and Bunnell, W. W The Presence of Heterophile Antibodies in Infectious Mononucleosis *Am J Med Sc*, 183 90–104, 1932.
12 Zarafonetis, C. J. D., Oster, H L, and Colville, V. F. Cold Agglutination of Sheep
13 M
14 Evans, A S. A Simplified "Qualitative" Method for Heterophile Antibody Determination Using Capillary Blood and a White Cell Pipette. *J. Lab. and Clin Med*, 32·1278–1281, 1947.
15 Tannen, J A Screening Test for Infectious Mononucleosis. *Am. J Clin. Path*, 23.
16 *U S Armed*
17 ious Mononucleosis *J. Hyg*, London, 49.471–481, 1951.

XV. Newcastle Disease

1. Burnet, F. M. Human Infection with the Virus of Newcastle Disease of Fowls. *M. J*
2.
3.
4
743, 1952.

2. Kingery, L B The Etiology of Common Warts Their Production in the Second Generation. *J. A. M A*, 76 440–442, 1921
3. Findlay, G M Warts Chapter 18 in *A System of Bacteriology in Relation to Medicine* Medical Research Council London His Majesty's Stationery Office, 1930, 7.252–258
4 [illegible]
5. [illegible]
6 [illegible]
7 Lyell, A, and Miles, J A R The Myrmecia A Study of Inclusion Bodies in Warts *Brit M J*, 1 912–915, 1951
8. Blank, H Virus-Induced Tumors of Human Skin (Warts, Molluscum Contagiosum) *Ann New York Acad Sc*, 54 1226–1231, 1952

3b Smithburn, K. C., Mahaffy, A. F., Haddow, A. J., Kitchen, S. F., and Smith, J. F. Rift Valley Fever Accidental Infections Among Laboratory Workers. *J. Immunol*, 62 213–227, 1949.
4. Smithburn, K. C., Haddow, A. J., and Gillett, J. D. Rift Valley Fever. Isolation of the Virus from Wild Mosquitoes. *Brit. J. Exper. Path.*, 29.107–121, 1948.
5 Smithburn, K. C, Haddow, A J., and Lumsden, W. H. R. Rift Valley Fever; Transmission of the Virus by Mosquitoes. *Brit. J. Exper. Path.*, 30.35–47, 1949.
6. Joubert, J. D. S, Ferguson, A L, and Gear, J. Rift Valley Fever in South Africa. The Occurrence of Human Cases in the Orange Free State, the North-Western Cape Province, the Western and Southern Transvaal. *South African M. J.*, 25 890–891, 1951.
7 Broom, J C, and Findlay, G M. Complement Fixation in Rift Valley Fever. *Lancet*, 1 609–611, 1932.

XVII. Rubella

1 Rake, G. Rubella. Chapter XXIII in *Viral and Rickettsial Infections in Man*, 2d ed. Edited by T M Rivers Philadelphia·J. B. Lippincott Company, 1952, pp. 484–488
2 Hiro, Y, and Tasaka, S Die Röteln sind eine Viruskrankheit. *Monatschr f Kinderh*, 76 328–332, 1938.
3 Habel, K Transmission of Rubella to Macacus Mulatta Monkeys. *Pub. Health Rep*, 57 1126–1139, 1942.
4 Anderson, S G Experimental Rubella in Human Volunteers. *J. Immunol*, 62·29–40, 1949.

XVIII Salivary Gland Virus Infection, Generalized

1 Smith, M G, and Vellios, F Inclusion Disease or Generalized Salivary Gland Virus Infection. *Arch. Path*, 50 862–884, 1950.
2 Kuttner A G, and T'Ung, T Further Studies on the Submaxillary Gland Viruses of Rats and Guinea Pigs *J Exper. Med*, 62 805–822, 1935.

XIX. Varicella

1. Stokes, J, Jr Varicella-Herpes Zoster Group Chapter XXVII in *Viral and Rickettsial Infections of Man*, 2d ed. Edited by T M Rivers. Philadelphia J. B. Lippincott Company, 1952, pp. 506–512.
2 Nagler, F P O, and Rake, G The Use of the Electron Microscope in Diagnosis of Variola, Vaccinia, and Varicella. *J Bact*, 55 45–51, 1948
3 Rake, G, Blank, H, Coriell, L L, Nagler, F. P O, and Scott, T. F. McN. The Relationship of Varicella and Herpes Zoster Electron Microscope Studies *J. Bact.*, 56 293–303, 1948.
4. Rivers, T M Varicella in Monkeys Nuclear Inclusions Produced by Varicella Virus in the Testicles of Monkeys *J Exper. Med*, 45 961–968, 1927.
5 Goodpasture, E W, and Anderson, K Infection of Human Skin, Grafted on the Chorioallantois of Chick Embryos, with the Virus of Herpes Zoster. *Am. J. Path*, 70 447–455 1944
6
7
8. Weller, T. H.
rived from
340–346, 1953.

XX. Warts

1. Wile, U. J, and Kingery, L B The Etiology of Common Warts Preliminary Report of an Experimental Study. *J A. M. A*, 73 970–973, 1919.

RICKETTSIAL DISEASES

JOSEPH E. SMADEL, M.D.*

Chief, Department of Virus and Rickettsial Diseases
Army Medical Service Graduate School
Walter Reed Army Medical Center
Washington 12, D C

* Present address, National Institutes of Health, Bethesda, Md.

TABLE 1

RICKETTSIAL DISEASES OF MAN

Group	Disease		Geographic Distribution	Natural Cycle		Transmission to Man	Serologic Diagnosis	
	Type	Agent		Arthropod	Mammal		Weil–Felix Reaction	Complement Fixation
Typhus	Endemic	*R. mooseri*	World wide	Flea	Small rodents	Infected flea feces into broken skin	Positive OX-19	Positive group and type specific
Typhus	Epidemic	*R. prowazeki*	World wide	Body louse	Man	Infected louse feces into broken skin	Positive OX-19	Positive group and type specific
Typhus	Brill's disease	*R. prowazeki*	U S A Europe	Recurrence years after original attack of epidemic typhus			Usually negative	Positive group and type specific
Typhus	Scrub	*R. tsutsugamushi*	Asia Australia Pacific Islands	Trombiculid mites	Small rodents	Mite bite	Positive OX-K	Positive in about 50% of patients
Spotted Fever	Rocky Mountain spotted fever	*R. rickettsi*	Western Hemisphere	Ticks	Small wild rodents, dogs	Tick bite	Positive OX 19 OX-2	Positive group and type specific
Spotted Fever	African tick fever	*R. conori*	Mediterranean and Africa	Ticks	Small wild rodents, dogs	Tick bite	Positive OX 19 OX-2	Positive group and type specific
Spotted Fever	Rickettsialpox	*R. akari*	North Atlantic Seaboard States Russia	Bloodsucking mite	House mouse	Mite bite	Negative	Positive group and type specific
Q Fever		*R. burneti*	World wide	Ticks	Small mammals, cattle, sheep, goats	Inhalation of dried infected material	Negative	Positive
Trench Fever		*R. quintana*	Europe Ethiopia Mexico	Body louse	Man	Infected louse feces into broken skin	Negative	None available

I. INTRODUCTION

A. General Remarks

The rickettsial infections of man fall into four categories—typhus, spotted, Q, and trench fevers. The typhus-spotted fever group is composed of several diseases each of which is caused by a different microorganism of the family *Rickettsiaceae.* The rickettsiae, about the size of bacteria, usually appear microscopically in stained smears as pleomorphic coccobacillary organisms; they are obligate intracellular parasites, and on the biologic scale lie between the bacteria and the viruses Each rickettsial agent is capable of multiplying in one or more arthropods and, except for the organisms of epidemic typhus and trench fever, each has one or more small animal hosts that serve as reservoirs in nature. The broad spectrum antibiotics provide excellent specific therapy for human infections caused by this family of microbes.

Table 1 lists the common rickettsial diseases of man and their etiologic agents and provides additional pertinent information. For more details, especially clinical, the reader is referred to appropriate chapters in the following textbooks: *Text Book of Medicine,* Cecil and Loeb; *Principles of Internal Medicine,* Harrison and others; and *Viral and Rickettsial Infections of Man,* Rivers and others.

B Precautions for Workers

In the early period of research on rickettsiae a number of investigators died from the rickettsial diseases they studied, and practically all laboratory workers in this field have suffered infections with these agents. The mortal hazard connected with handling these organisms has dimin-

man[3] With proper equipment, training, and vigilance practically all of these special hazards can be controlled The small laboratory that occasionally does rickettsial diagnostic work, however, is incapable of proper precautionary procedures and hence should refuse to undertake diagnostic isolation of rickettsiae from clinical materials Since serologic diagnostic procedures that employ noninfectious antigens, obtainable commercially, provide the physician with earlier definitive information than do the more laborious isolation studies, such practice is entirely justified as regards service to the clinician who refers material to the laboratory.

II. ISOLATION AND IDENTIFICATION OF RICKETTSIAL AGENTS

Before attempting diagnostic isolation of a rickettsial agent from a given patient, the laboratory worker should reread paragraph I, B and then decide whether the undertaking, if successful, would provide useful information in the particular instance that could not be supplied more safely, quickly, and accurately by appropriate serologic studies on specimens of blood taken at the proper times during the course of the patient's disease. The following paragraphs are written for those instances when the laboratory processes infectious material either for diagnostic work or during the course of research studies It is assumed that the reader is familiar with the general procedures employed in a bacteriologic laboratory, including aseptic technics used for autopsying small animals and for preparing tissue suspensions Hence, detailed instructions regarding procedures will be given only when the technology is unusual or pertinent to the immediate problem

A Collection, Shipment, and Inoculation of Materials

1. *Human specimens* Each of the diseases in this group is characterized by rickettsemia during the febrile period of the illness in man, and blood provides the most accessible and suitable material for isolation studies Under field conditions one sometimes inoculates animals at the patient's bedside with unclotted whole blood immediately after its withdrawal *Such disruptions of ward routine can generally* be avoided since the specimen is suitable if injected into animals within a half hour after venesection. Under such circumstances, the freshly drawn blood is immediately defibrinated or heparinized (merely wetting the syringe and needle with sterile USP heparin solution prior to venipuncture is

ished appreciably within the last few years as a result of (*a*) recognition of defects in common laboratory procedures and introduction of simple safety measures, (*b*) development of potent vaccines, and (*c*) discovery of antibiotics with specific therapeutic action. At the Army Medical Service Graduate School (AMSGS) great stress has been and still is placed on safe laboratory methods.[1] Until a few years ago all personnel in the rickettsial laboratory were given a basic course of immunization against epidemic typhus, murine typhus, Q fever, and Rocky Mountain spotted fever; furthermore, periodic booster vaccinations were given so that each individual maintained in his blood demonstrable complement-fixing antibodies for epidemic and murine typhus and Q fever. More recently the vaccination program has been less rigorously imposed, and increasing emphasis has been placed on early recognition and prompt treatment of laboratory infections. Primary vaccination against the agent or agents under intensive study by the individual worker is, however, still employed, and one or more booster doses are given at appropriate intervals. *With the increased dependence on chemotherapy to*, prevent serious illness and an undue period of absenteeism, special indoctrination of laboratory personnel is essential. Each worker is trained to report promptly to the designated physician, who is responsible for the group, if fever and headache develop and persist for 24 hours. If the physician finds no obvious cause for the illness at this time, he (*a*) assumes that a rickettsial infection is developing, (*b*) takes the appropriate specimens which will subsequently assist in establishing the etiologic diagnosis, and (*c*) immediately institutes specific therapy. Alertness on the part of the laboratory worker and the physician is mandatory.

Barring the obvious accidents, the well-trained worker in a carefully regulated laboratory may undertake the simpler type of rickettsial studies with reasonable chance of avoiding infection in himself and others. Certain types of procedures are particularly dangerous, however, these are connected with (*a*) work with experimentally infected embryonated eggs and naturally or experimentally infected arthropods, (*b*) the use of the Waring blendor, (*c*) employment of the intranasal route for inoculating animals and the intrarectal route for inoculating arthropods, for example, lice, and (*d*) use of animals which excrete rickettsiae or contaminate their ectoparasites. Examples of (*d*) are guinea pigs that excrete *Rickettsia burneti* in their urine[2] and mice that in one laboratory were suspected of infecting their ectoparasites *with R. burneti, which in turn infected*

species other than those mentioned have been used by different investigators for primary isolation of strains, but for ordinary diagnostic studies in the United States the guinea pig and the mouse are adequate. Particular mention should be made here of the embryonated egg. The chick embryo is one of the most valuable hosts available for work when employing egg-adapted strains of rickettsiae in problems of research and in commercial production of vaccines and antigens, nevertheless, it leaves much to be desired as a tool for primary isolation of these micro-organisms. The experience of Rose[5] in recovering *R akari* from the blood of 8 of 10 patients under study when mice were used, in contrast to a single isolation when portions of the same specimens were inoculated into embryonated eggs, is in accord with experience at the AMSGS with eggs for primary isolation of *R tsutsugamushi*, *R mooseri*, and *R. rickettsi*.

2. *Arthropods and animal reservoirs*. Isolation of rickettsiae from naturally infected arthropods and small wild rodents seldom falls within the sphere of the clinical rickettsial diagnostic laboratory. If special investigations of the epidemiology and ecology of an unidentified disease suspected of being of rickettsial origin are indicated, then the laboratory worker should collaborate with an epidemiologist and an entomologist interested in such a joint study. The following paragraphs indicate a general line of approach for the laboratory worker.

The resources of a research laboratory are generally required to identify a rickettsial agent recovered from naturally infected arthropods or rodents. The steps in the complete identification procedure are mentioned in Chart 1 and discussed in paragraph II, E It should be stated here, however, that such isolation attempts are beset with many problems other than those concerned directly with rickettsiae. In such work one encounters the gamut of microbial agents capable of causing natural infections in the host under study, and identification of these is often more worrisome to the laboratory worker than is the recognition of the rickettsiae which he seeks.

a. Arthropods. In studying stained smear preparations of the tissues of various species of arthropods, one frequently observes objects which are morphologically indistinguishable with certainty from pathogenic rickettsiae. Hence, such morphologic evidence is not sufficient to enable a critical diagnosis of infection with pathogenic rickettsiae, and the proper handling of the problem involves the following steps: (1) living lice, fleas, ticks, or mites are collected and placed in cotton-plugged

adequate) and promptly brought to the laboratory for inoculation. Specimens obtained during the 1st week of illness may be injected forthwith; however, later specimens should be freed of plasma which contains antibody For this purpose plasma is removed from the centrifuged erythrocytes and buffy coat; the cellular elements are resuspended in an equal volume of physiologic saline and inoculated into animals. Freshly clotted specimens of blood from febrile patients are sometimes employed for isolation studies; here the serum is removed and saved for future serologic studies, and the clot after being ground with diluent serves as the inoculum. Occasionally isolation attempts are warranted on other types of clinical material, that is, sputum or urine[4] from patients with Q fever or tissues obtained under aseptic conditions at autopsy. The addition of one of the soluble salts of penicillin in sufficient amounts to bring the concentration to 100 units per ml. of inoculum is indicated with urine or sputum in order to suppress bacterial infection in the animals. Streptomycin and the broad spectrum antibiotics should not be used because of their appreciable rickettsiostatic effect.

When the patient providing the material for isolation studies is situated more than a half hour's travel from the diagnostic laboratory the specimens should be frozen and maintained in dry ice. For this, whole blood is shell-frozen in adequate-sized tubes or bottles by immersion in an alcohol-dry ice mixture and shipped in an insulated container with sufficient dry ice to maintain the specimen in the frozen state. If facilities permit, however, it is preferable to obtain 12 to 15 ml. of blood, centrifuge it promptly after clotting, transfer the serum to a separate container, and then freeze and ship the samples of serum and blood clot. The diagnostic laboratory may use a 10 per cent suspension of the ground clot as inoculum for isolation attempts and may save the serum as an acute phase sample for future serologic tests along with the convalescent serum. Specimens other than blood submitted for isolation attempts should also be shipped in dry ice. Frozen specimens containing rickettsiae should be thawed rapidly by rotating the tube containing the material in a beaker of tap water and then promptly processed and injected into animals.

The two species of animals which are ordinarily employed for primary isolation of rickettsiae of the typhus, spotted, and Q fever groups of diseases are adult male guinea pigs and adult white mice that are injected intraperitoneally with 2 to 4 ml. and 0.5 ml., respectively, of the specimen. A variety of animals is susceptible to these agents, and several

days in order to permit reactivation of rickettsiae that may be present. For this 10 to 15 ticks are placed in a cage on the clipped skin of the abdominal wall of a guinea pig The cage is made by perforating the metal top of a mailing tube[7] and by attaching the top to the guinea pig by adhesive tape. The ticks are removed as they detach, accumulated in a container at room temperature, and finally pooled for trituration and inoculation of animals. It must be remembered that the tick feces may contain viable *R rickettsi, R conori, R burneti,* or *Bacterium tularense,* and that these organisms may be transmitted to the guinea pig on which the ticks feed

If arthropods are to be transported alive, one must prevent desiccation, starvation, and exposure to extremes of temperature. Since lice and fleas require feeding at least once daily, it is seldom practical to ship them for long distances Ticks, which are much more resistant to starvation and desiccation than most other vectors, may be shipped in cork-stoppered tubes.

It is wise to refrigerate a parcel containing live arthropods upon its arrival at the diagnostic laboratory, this is done *before* the parcel is opened, in order to immobilize arthropods that may have escaped from a possibly broken container within the parcel Furthermore, chilling at 3° to 5° C is the simplest method for immobilizing arthropods during the preparation of a suspension for inoculation Before grinding, arthropods may be washed with successive portions of sterile distilled water or normal saline in order to reduce the numbers of micro-organisms contaminating their exterior surfaces. Chemical germicides or detergents are not recommended for this since traces may remain on the arthropods despite repeated washings and may kill rickettsiae when the arthropods are triturated

b Rodents Spleen removed aseptically from small rodents suspected of being infected with a rickettsial agent provides suitable material for isolation studies. A suspension of the tissue may be prepared and inoculated immediately into animals or the whole organ may be stored at −70° C until a convenient time for the isolation attempt.

B. Manifestations of Infection in Inoculated Animals

The two animals of choice for isolation of rickettsiae in connection with diagnostic work are guinea pigs and mice. The clinical and pathologic findings in these species are mentioned in Chart 1 and discussed in some detail in the following paragraphs

or screw-capped containers (rubber-stoppered tubes are not desirable); (2) a representative sample is preserved in 70 per cent alcohol for identification by an entomologic taxonomist; (3) a sample of the collection is preserved for future microbiologic work by rapidly freezing the intact arthropods in a tightly stoppered container, which is stored at -70° C.; (4) a suspension for animal inoculation is prepared by grinding the living arthropods or the quickly thawed material from (3) in a suitable diluent usually containing penicillin (see paragraph II, A, 1) and removing gross particles by light centrifugation; and (5) the suspension is tested in animals as outlined for specimens of human blood. Certain points concerning the handling of living arthropods and the preparation of inocula merit further consideration, since a successful recovery of rickettsiae often requires attention to details.

One may maintain arthropods alive for several days to permit possible multiplication of rickettsiae In the absence of safe and simple technics, the maintenance of potentially infected fleas or of such mites as *Allodermanyssus sanguineus* or larval chiggers is not recommended for a diagnostic laboratory. Ticks and human body lice can be kept with relative ease and safety if the operator accounts for each individual tick or louse and if simple precautions are taken to prevent contamination of the laboratory by potentially infectious arthropod feces. Lice should be fed at least once and preferably twice daily. A normal rabbit is a suitable host.[8] The lice are kept in a beaker on a felt pad which is covered on one side with scotch tape; they cannot climb upward on a clean glass surface. Between feedings this beaker is placed in a desiccator jar in which the relative humidity is kept at approximately 60 per cent by a mixture of sulfuric acid and water of specific gravity 1.295. The entire desiccator jar is incubated at 30° to 32° C. During 1 week of daily feedings and incubation in this range of temperature, epidemic typhus rickettsiae in lice multiply to demonstrable levels, at which time the insects may be sacrificed for animal inoculation. *R prowazeki* causes an infection in lice that is eventually fatal for the insects. Infected lice may pass feces which contain considerable numbers of viable rickettsiae, and the organisms maintain their viability for several weeks under some conditions of temperature and humidity. Hence, the skin of the rabbit must be treated with 70 per cent alcohol after each feeding, and the felt pad and beaker must be handled with care to prevent the dustlike dried feces from causing accidental infections in personnel.

Engorged ticks are suitable for immediate use in isolation attempts intended to detect *R. rickettsi*. Unfed ticks should be fed for several

days in order to permit reactivation of rickettsiae that may be present. For this 10 to 15 ticks are placed in a cage on the clipped skin of the abdominal wall of a guinea pig. The cage is made by perforating the metal top of a mailing tube[7] and by attaching the top to the guinea pig by adhesive tape. The ticks are removed as they detach, accumulated in a container at room temperature, and finally pooled for trituration and inoculation of animals. It must be remembered that the tick feces may contain viable *R. rickettsi*, *R. conori*, *R. burneti*, or *Bacterium tularense*, and that these organisms may be transmitted to the guinea pig on which the ticks feed.

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B. Manifestations of Infection in Inoculated Animals

The two animals of choice for isolation of rickettsiae in connection with diagnostic work are guinea pigs and mice. The clinical and pathologic findings in these species are mentioned in Chart 1 and discussed in some detail in the following paragraphs

CHART 1
PRIMARY ISOLATION OF RICKETTSIAE

BLOOD FROM FEBRILE PATIENT
2–4 ml.
intraperitoneally
TWO MALE GUINEA PIGS, 450 gm.

No Fever	Fever with		
	No Scrotal Reaction	*Scrotal Swelling* *Suspect*	*Scrotal Necrosis*
Discard as negative on 21st day or bleed at 28 days and test for specific complement-fixing antibodies.	a) *R. prowazeki* b) *R. burneti* c) Some strains of *R. tsutsugamushi*	a) *R. mooseri* b) *R. conori* c) Some strains of *R. rickettsi* d) Some strains of *R. akari*	a) *R. rickettsi*

Maintain by passage of bacteriologically sterile material. Demonstrate intracellular rickettsiae in Giemsa or Macchiavello stained smears of appropriate tissues.

Final Identification

1. Demonstration of antibody in sera of convalescent guinea pigs using [illegible]
2. [illegible]
3. [illegible] of specific complement-fixing antigen for testing with standardized positive antisera.

BLOOD FROM FEBRILE PATIENT
0.5 ml.
intraperitoneally
SIX ADULT WHITE MICE

No Clinical Disease		*Sickness, Ascites, and Death*
Autopsy one mouse on 14th day, another on 21st		Intracellular rickettsiae in stained smears of peritoneal and splenic surfaces *Presumptive* *R. tsutsugamushi* or *R. akari* Maintain by passage *Final Identification* 1. Demonstration of resistance of convalescent mice to known strain: reciprocal cross-protection tests if indicated 2. Infect guinea pigs and test convalescent serum for specific antibodies. 3. Establishment of isolate in eggs and preparation of soluble antigen for testing with standardized positive antisera.
Autopsy four surviving mice on 28th day and discard. Report negative results if all animals normal	If minimal peritonitis and splenomegaly observed at autopsy, suspect *R. tsutsugamushi* or *R. akari* of low virulence, or *R. burneti* or *R. mooseri* Maintain for several passages, look for rickettsiae in smears. If rickettsiae observed and if strain becomes lethal for mice do procedures listed under Final Identification. If rickettsial infection remains nonlethal for mice, challenge convalescent mice with known lethal strains of *R. tsutsugamushi* or *R. akari* If *R. burneti* or *R. mooseri* considered likely, establish in guinea pigs and test their convalescent sera for complement-fixing antibodies.	

1. *Guinea pigs.* Rickettsial infection in the guinea pig is almost invariably accompanied by fever, which usually appears during the latter half of the 1st week after inoculation with materials from patients with murine typhus or Rocky Mountain spotted fever, or early in the 2d week after injection with blood from cases of epidemic typhus or Q fever (Table 2). Rectal temperatures of 40° C or 104° F, or higher, are indicative of fever. Infected animals remain febrile from a few days to a week, after which those inoculated with *R prowazeki* or *R. mooseri* recover without sequelae. In contrast, even primary isolates of *R. rickettsi* and *R burneti* frequently produce fever of a week's duration and are sometimes lethal; after a few passages they may kill the majority of guinea pigs

The response of the scrotal tissues of adult male guinea pigs during rickettsial infection aids in preliminary identification of the agent. The three classical responses are those encountered in epidemic typhus, murine typhus, and Rocky Mountain spotted fever In epidemic typhus no abnormalities of the scrotum are observed throughout the experimental disease The reaction in murine typhus is characterized by edema and erythema, which begins about the time fever develops The skin becomes glossy, tightly stretched, and red, and the testicles are not readily pushed back into the abdominal cavity The abnormalities subside after a few days. The most severe reaction occurs in experimental spotted fever This begins after several days of fever, with findings at the onset similar to those of murine typhus However, a macular rash subsequently appears over the scrotum and adjacent areas of skin, and within a day or so becomes purpuric The lesions become necrotic, and superficial ulcers appear that heal with scarring after some days if the animal survives The scrotal reactions seen in other rickettsial infections are indicated in Chart 1.

Post-mortem examination of infected guinea pigs that are sacrificed after several days of fever reveals few abnormalities other than those associated with mild peritonitis and the scrotal reaction, if present Evidence of the former may be limited to a thin, fibrinous exudate over the splenic surface The scrotal reaction when present is characterized by edema, injection, and hemorrhages in the tunica vaginalis, which is brought into view by applying gentle traction to the spermatic cord until the testicle is retracted into the exposed abdominal cavity.

Smears for microscopic examination are prepared by lightly touching the uncut surface of the spleen or the tunica vaginalis to a thoroughly

cleaned glass slide, bloody areas of tissue are avoided. The smear may be fixed and stained by Giemsa's method, in which case the intracellular rickettsiae appear as bluish purple, small, coccobacillary structures. Macchiavello's procedure is *generally preferred by the author, however*, since it stains the rickettsiae bright red in sharp contrast to the blue background material Snyder[8] summarizes the method as follows:

[illegible]

which is then quickly dipped in the freshly prepared citric acid solution; *it is* removed immediately and placed in a dish containing running tap water. The final step is the flooding of the slide with methylene blue, which is poured off after a few seconds The slide is then washed briefly in running tap water, and dried with a piece of filter paper.

Rickettsiae are usually scarce in smears from animals inoculated with material for diagnosis, and a few words of advice regarding the method of microscopic examination are in order The stained smear *should be scanned under low power magnification until one* finds a thin portion in which there are several large, properly stained serosal cells. These cells, and a few others in the immediate area, are examined under oil immersion. If rickettsiae are not observed immediately one returns to the low power objective and searches for another likely area. Thus, most of the allotted time is spent looking for suitable cells in which to see rickettsiae, and high magnification is employed only to determine whether they are actually present. In original and passage animals, microscopic identification of rickettsiae is established only when the organisms are observed intracellularly.

2. *Mice.* Mice inoculated with clinical material containing virulent strains of *R tsutsugamushi* or *R. akari* usually show signs of illness toward the middle or end of the 2d week. Roughening *of the fur and inactivity* are followed in a few days by abnormalities in breathing and abdominal fullness If death occurs at this time the pathologic changes may be minimal, but usually the beginning stages of the manifestations described *below are recognizable* Mice with *severe* scrub typhus that survive into the 3d week often display obvious subcutaneous edema of dependent parts, marked ascites, and labored rapid respirations. Such animals at autopsy show wet subcutaneous tissue with congestion of vessels and enlargement of superficial lymph nodes. The peritoneal cavity

is distended with 3 to 8 ml of serofibrinous fluid The spleen is several times normal size and the liver congested The pleural cavities contain up to several mls. of fluid, and areas of hemorrhagic pneumonia may occur. Two types of smears for microscopic demonstration of rickettsiae are prepared One is an impression smear from the surface of the spleen. The other consists of scrapings obtained from the parietal peritoneum with the edge of a knife blade and spread thinly over a clean glass slide. Giemsa's procedure is usually used for staining such smears Advice regarding microscopic examinations of rickettsial smears was given in paragraph II, B, 1

Rickettsiae of low virulence for mice, including many newly recovered strains of *R akari* and *R tsutsugamushi* and practically all strains of *R burneti* and *R. mooseri*, produce minimal signs of disease which may

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rickettsiae in stained smears and to initiate passage of tissues to normal mice.

C MAINTENANCE OF AGENTS IN ANIMALS

Most of the pertinent information on maintenance of newly recovered strains of rickettsiae is given in Chart 1 and Table 2. In general, one of the sick animals in the group receiving clinical material is sacrificed within a few days after onset of obvious disease. Its spleen, tunica, or brain is removed aseptically, ground in a mortar with abrasive, and made up to approximately a 10 per cent suspension with an appropriate diluent After freeing the suspension of gross particles by permitting the material to stand for a few minutes or by low speed centrifugation, 1 to 2 ml are promptly injected intraperitoneally into each of 2 or 4 guinea pigs, or 0 25 to 0 50 ml amounts are administered intraperitoneally to 4 or 6 mice Material from guinea pigs is passed to guinea pigs and from mice to mice until the agent is established in a laboratory host.

One of the 2 original guinea pigs injected with clinical material and 3 or 4 of the mice should be kept under observation for some days after a member of the group has been sacrificed for passage. This provides another source of infectious material in case routine bacterial cultures reveal contaminants in the suspension used for passage Moreover, observations on the course of the disease in surviving animals of the original group may assist in preliminary identification of the agent (Chart 1). Finally, sera from convalescent guinea pigs of the original or 1st passage

TABLE 2

MAINTENANCE OF RICKETTSIAE IN GUINEA PIGS OR MICE

Rickettsial Agent	Animal of Choice	Original Animals						Passage Animals		
		Course of Disease		Material Used for Next Passage						
		Incubation *Period*	Clinical *Manifestations*	*Time Collected*	Tissue and Concentration	Route Injected		Incubation Period	Fatal Outcome	No *Without* Obvious Disease
Prowazeki	Guinea pig	5–12 days	Fever	2d–3d day of fever	Spleen or brain 10%	IP		6–8 days	Never	Few
Mooseri	Guinea pig	3–10 days	Fever, scrotal swelling	2d–3d day of fever	Tunica or spleen 10%	IP		3–5 days	Never	Few
Rickettsi	Guinea pig	3–10 days	Fever; scrotal necrosis	2d–3d day of fever	Spleen or tunica 10%, whole blood	IP		3–5 days	Usual	Few
·onori	Guinea pig	3–6 days	Fever, scrotal swelling	2d–3d day of fever	Tunica 10%	IP		3–4 days	Rare	Few
·urneti	Guinea pig	5–12 days	Fever	2d–3d day of fever	Spleen 10%	IP		3–6 days	Sometimes	Rare
..kari	Mouse	6–9 days	Rough fur, inactivity	Few days after onset	Spleen 10%	IP		4–5 days	Usual	Rare
Tsutsugamushi	Mouse	6–14 days	Rough fur, inactivity, ascites	Few days after onset	Spleen 10%	IP		5–10 days	Usual	Rare

group when tested for type-specific antibodies may provide the definitive identification of the agent. Specific antibodies may appear even though clinical disease was unrecognized in "misses" among animals in the original group and usually develop in the occasional passage animal that survives without apparent illness while its mates develop disease. Surviving convalescent mice when subjected to challenge with established strains of *R. akari* or *R tsutsugamushi* may also assist in early identification of the agent

Blind passage is usually unrewarding when all guinea pigs inoculated with a given clinical material remain well. If strong suspicion exists that inapparent infection occurred in the guinea pigs, then the laboratory worker should bleed the animals at the end of 4 weeks of observation and test the sera for specific antibodies.* In contrast, blind passage, or rather passage on the basis of questionable evidence of disease, is often worth while in mice inoculated with specimens from patients with rickettsialpox or scrub typhus.

Several practices of ancillary importance in maintaining newly isolated rickettsial agents are epitomized below. Physiologic saline solution or peptone broth may be used as diluent when 10 per cent suspensions of infected tissue are employed for passage However, a diluent which is less damaging to rickettsiae—for example, saline with sufficient normal rabbit or guinea pig serum to make a 10 per cent concentration—is usually employed. Each inoculum is cultured for contaminating bacteria, for this purpose a portion is streaked on blood agar and a few drops are placed in thioglycollate broth A portion of the tissue used for passage to animals is stored at -70° C , a bit of freshly removed sterile organ is placed in a properly labeled, stoppered tube and stored in a dry-ice chest

D Cultivation of Rickettsiae in Embryonated Eggs

1 *General.* Each of the rickettsiae listed in Table 1, except *R. quintana*, can be adapted to luxuriant growth in the embryonated egg The process usually is either tedious or difficult, however, which accounts for the fact that the embryonated egg is held in poor esteem for the primary isolation of rickettsiae from clinical material. For the same reasons the preparation of complement-fixing antigen from yolk sacs infected with a newly isolated strain is placed last in the lists of procedures men-

* Price (*Science*, 1953, *118*, 49) has demonstrated the occurrence in naturally infected ticks of strains of *R rickettsii* which are nonpathogenic for guinea pigs but grow readily in embryonated eggs Such strains may elicit an immunogenic response in guinea pigs.

tioned in Chart 1 for final identification of a strain. When adapting a rickettsial strain to growth in embryonated eggs, the most infectious material available from experimental animals (Table 2) is obtained under strictly aseptic conditions and inoculated without antibiotics into the yolk sacs of 5- to 7-day-old embryos. The strain is maintained by passage of yolk sac material into new embryonated eggs at intervals of 6 to 10 days. From a few to many such passages may be required before a constant fatality rate is manifested in inoculated eggs and before rickettsiae are demonstrable in stained smears of yolk sac tissues. It may be mentioned that concentrations of the order of 10^6 rickettsiae per gm. of yolk sac tissue are required in order to recognize with assurance the organisms in stained smears prepared in the usual manner. Moreover, yolk sac tissues which provide barely adequate starting material for preparation of complement-fixing antigens contain at least 10^8 rickettsiae per gm. It is not unusual under optimal conditions with well-adapted egg lines of a number of the agents to obtain 10^{11} infectious units per gm. of yolk sac. However, *R. tsutsugamushi* and *R. rickettsi* grow less luxuriantly, and titers of 10^{-8} to 10^{-9} are the rule with the former. The procedures used for cultivation of rickettsiae in the yolk sac of embryonated eggs will be given in some detail in the following subsection. Maintenance of certain strains by other routes in eggs—for example, on the chorioallantoic membrane—and cultivation in tissue cultures present no particular advantages to the diagnostic laboratory over the yolk sac route; hence, will not be discussed.

2 *Incubation and inoculation.* Fertile hen's eggs from flocks free of pullorum and Newcastle disease are kept at 37° to 38° C. in a commercial egg incubator where temperature and humidity (about 60 per cent) are properly controlled After 5 to 7 days of incubation, eggs are candled and those with viable embryos are selected for inoculation with rickettsial material. The eggs are placed with the small end down in boards with properly bored holes (Fig. 1). The large end containing the air sac is painted with tincture of iodine, and a small hole is made through the shell, using a motor-driven or hand drill, after which dust is wiped off with an alcohol swab. The inoculum is loaded into a syringe to which has be

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are injected. Experience has shown that under these conditions the inoculum is almost invariably placed in the yolk sac. The needle is withdrawn perpendicularly while one finger fixes the barrel of the syringe to prevent inoculum dripping from the needle during movement to the next egg After all the eggs have been inoculated the ends are wiped with alcohol and sealed with wax, collodion, or scotch tape The eggs are then placed in trays in an egg incubator held at 35° C When small numbers of embryonated eggs are used only occasionally, simpler equipment than that mentioned above may be employed for incubation Here a bacteriologic incubator set at 37° C and containing a pan of water will serve for incubating eggs before and after inoculation Furthermore, the ordinary cardboard package in which eggs are purchased at the grocery can be used in place of special trays

Inoculated eggs are candled daily. If a good commercial candler is unavailable, one may substitute a microscope lamp fitted with a cardboard diaphragm having a hole into which the large end of the egg can be inserted to a depth of a centimeter

3. *Harvest, passage, and storage* Embryos that die within 24 to 48 hours after inoculation are discarded When candling after this period reveals sluggish movements of the embryo and inadequate circulation, as evidenced by puddling of blood in dependent parts of the membranes, the egg is opened and examined The arrangement shown in Figure 2 is convenient for such work The egg is placed in an individual holder (an ether or juice can with the top removed and the rim padded with plasticine), and the air sac end is painted with tincture of iodine The shell over the air sac is cracked by a sharp blow with forceps and then removed piecemeal. The exposed shell membrane which forms the floor of the air sac is torn away with sterile forceps The egg contents are then poured into a sterile petri dish, another pair of sterile forceps may be used to assist the delivery. With two pairs of forceps the yolk sac tissue is detached from the embryo, gently squeezed free of excess yolk fluid, and then transferred to another petri dish The same forceps are employed to separate a small bit of tissue from the yolk sac membrane. This is smeared around on a dry portion of the petri dish to remove excess fluid, after which it is smeared on a glass slide, heat-fixed, and stained by Macchiavello's technic. The slide is examined immediately under oil immersion to determine whether bacteria or rickettsiae are present; cocci and bacilli usually stain blue while rickettsiae are red. If no bacteria are observed the worker returns to the bench and separates the yolk sac

tissue into equal parts; one half is placed in a mortar preparatory to grinding and the other half in a Wassermann tube for storage at –70° C. The portion in the mortar is triturated with alundum, and sufficient buffered saline solution added to make an approximate 10 per cent suspension, which is taken up in a pipette with a sucking tube attached and dispensed into a centrifuge tube. The final drop of suspension is employed for culture on a blood agar plate. After centrifugation at low speed the midzonal fluid is used as inoculum for eggs of the next passage.

E. Serologic and Immunologic Identification of Agents

The first and simplest step in the final identification of a newly [illegible] one suspects the isolate belongs (Chart 1). The technic of the test is discussed in paragraph III, C of this chapter. Certain of the type-specific rickettsial antigens can be obtained commercially. If these are not readily available, the small laboratory would be well advised to enlist the help of one of the laboratories regularly engaged in rickettsial work. Submission of 2 to 3 ml. of sterile serum from 1 or several convalescent guinea pigs along with a brief description of the circumstances of the isolation and the experimental disease produced in animals should provide the reference laboratory with suitable material for preliminary identification. Such serologic identification of a rickettsial strain is generally adequate in clinical diagnostic work. The additional steps in identification listed in Chart 1—cross-immunity tests and preparation of specific complement-fixing antigens—are complicated, expensive, time-consuming, and unwarranted except in special circumstances.

Isolates encountered in inoculated mice and suspected of being *R. akari* or *R. tsutsugamushi* are readily identified by demonstrating resistance of convalescent animals to approximately 100 LD_{50} of a known strain of one of these agents* (Chart 1). Another procedure is to infect guinea pigs with the mouse-adapted isolate and then test their convalescent sera for specific complement-fixing antibodies. This is usually successful even though the original clinical specimen that yielded the rickettsiae in mice failed to produce obvious disease in guinea pigs.

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* Classical strains of the common rickettsial agents may be purchased from the Viral and Rickettsial Registry of the American Type Culture Collection, Washington, D.C.

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS OF RICKETTSIAL DISEASES

A. Collection and Shipment of Specimens

1. *Time of collection.* The laboratory diagnosis of rickettsial diseases is established most convincingly and easily by the demonstration of the appearance of specific antibodies in the blood of the patient and their increase in titer during the course of infection and convalescence. Several types of antibodies of diagnostic importance develop at different times during the disease. Therefore, the well-equipped laboratory should perform several types of tests designed to provide the earliest possible tentative laboratory diagnosis as well as the final specific laboratory diagnosis. Such a laboratory can be of most assistance to the clinician if he submits 3 samples of blood taken from the patient as follows: (1) during the first few days of illness, (2) during the 2d week, and (3) during the 4th week after onset Some information of diagnostic value can be obtained from a single specimen of blood taken late in the febrile period or during convalescence, but such limited studies are not to be encouraged It is the duty of the laboratory worker to educate the clinician to employ fully the available diagnostic tests to the best advantage for the patient, and this cannot be done when only 1 sample is submitted. If the laboratory worker is to employ his procedures intelligently, he must know at what period the blood was drawn. Therefore, the clinician must indicate this temporal relationship on the request slip which accompanies each sample submitted.

2. *Technic and shipping* Specimens for serologic studies are obtained and shipped as follows at least 5 ml of blood are drawn aseptically from the patient's vein with a sterile dry syringe and needle, placed [illegible] bearing the patient's name, the physician's name and address, the clinical diagnosis, the date of onset of disease, the date on which the sample was taken, and the type of test requested, is wrapped around the inner tin container before it is placed inside the outer cardboard container. The outside cardboard container should bear the return address of the physician and the address of the laboratory; it should also be clearly marked "Rush—Specimen for Bacteriologic Examination—Pouch with First-Class Mail" The attending physician should be cautioned against send-

ing specimens so that they reach the laboratory on week ends or holidays. It is preferable that he hold the specimen for a day or so in his refrigerator and then mail it to arrive on a workday.

B. Weil-Felix Test

1. *Preparation of proteus suspensions and technic of the test.* The Weil-Felix test continues to be of considerable importance in the early presumptive diagnosis of a number of rickettsial diseases.[9] While it is recognized that the reaction is nonspecific, nevertheless, its simplicity and the fact that positive results may be obtained late in the 2d week of disease warrant its use. The test tube titration of antibody is more accurate than is the slide agglutination method, and the use of the last-mentioned should be restricted to field workers. For details of the proteus slide agglutination technics of Castañeda[10] and of Welch,[11] the original references should be consulted. The procedures employed at the AMSGS for the preparation of suspensions of *Proteus bacillus* OX-19, OX-2, and OX-K strains, and their use in agglutination tests, have been described by Plotz[12] essentially as follows:

Only the O or nonmotile variant of the *Proteus bacillus* is used for the agglutination reaction since it is this antigen which reacts specifically with sera from rickettsial diseases The purity of the strain should be determined frequently by streaking on a veal agar infusion plate. After twenty-four hours' incubation, the smooth, nonspreading O type colonies are selected and transferred to tubes of dry agar and broth If the organisms are nonmotile the culture may be used for the agglutination test All cultures should be maintained on dry agar slants. Lyophilization is helpful in maintaining a culture as a pure O variant.

The agglutination reaction may be performed with living or killed cultures. The antigen is prepared by suspending eighteen to twenty-four hour agar cultures in 0.85 per cent saline The turbidity of the suspension is adjusted to that of tube 3 of the McFarland nephelometer scale. The killed antigen is prepared as follows. A smooth nonmotile strain is grown on agar in Kolle flasks, and the culture is washed down with sufficient saline to make a heavy suspension, and 0 5 per cent formalin is added. This represents the concentrated stock This antigen is then diluted with saline when needed to have a turbidity equal to tube 3 of the McFarland scale.

A macroscopic agglutination test is performed by thoroughly mixing 0 5 cc. of serum dilution and 0 5 cc. of antigen suspension Serum dilutions of 1 to 10 through 1 to 640 (final dilution 1 to 20 through 1 to 1,280) are usually sufficient A control tube containing 0 5 cc. of antigen and 0 5 cc of saline should be included as well as a positive serum control Tests and controls are incubated in the water bath at 37° C for two hours followed by storage overnight in the icebox. Complete agglutination is indicated by absolute clearing of the supernatant fluid and by settling of the organisms in large white particles at the bottom of the tube Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of

bacterial clumps When the tubes are shaken, granular agglutinated masses of bacteria are seen To facilitate reading, the tubes may be held in front of a concave mirror and the image examined

2. *Interpretation of results* The typical responses obtained with suspensions of OX-19, OX-2, and OX-K Proteus organisms and convalescent sera from patients with rickettsial diseases are given in Table 3 The classical findings of agglutination of the OX-19 organism by sera from cases of epidemic and murine typhus and of OX-K suspensions by sera from patients with scrub typhus are indicated The Weil-Felix response in Rocky Mountain spotted fever may be of several types, a high OX-19 and a low OX-2, or elevation of both OX-19 and OX-2, or, occasionally, a low OX-19 and a high OX-2 [13] Sera of cases with rickettsialpox or Q fever to agglutinate any of the three organisms

TABLE 3
USUAL WEIL-FELIX AGGLUTINATION REACTIONS OBSERVED IN RICKETTSIAL DISEASES

	OX-19	OX-2	OX-K
Epidemic typhus	++++	+	0
Murine typhus	++++	+	0
Scrub typhus	0	0	+++ +
Rocky mountain spotted fever and	++++	+	0
African tick fever	+	++++	0
Rickettsialpox	0	0	0
Q fever	0	0	0

The Weil-Felix agglutinins may appear as early as the 5th or 6th day after onset of fever in those diseases in which the reaction becomes positive, and they are almost always present by the 12th day. These antibodies generally reach their maximum in early convalescence and then decline rather rapidly to nondiagnostic levels in 1 to several months. Occasional patients with typhus or spotted fever do not develop OX agglutinins This occurs in about 15 per cent of vaccinated persons who subsequently contract typhus [14] and is the rule in Brill's disease, particularly when the recurrent attack occurs within a decade after the initial episode.[15,16]

A rise in antibody titer, demonstrated in a series of 2 or more sera, is essential for the presumptive diagnosis of rickettsial disease when the

Weil-Felix reaction is employed. One is not justified in interpreting the results of tests on a single serum, unless the titer is high, that is, well above 1/160. The Weil-Felix reaction is of no value in differentiating epidemic and murine typhus and frequently fails to provide even presumptive evidence for separating spotted fever from murine typhus.

The importance of maintaining a supply of known positive human sera for rechecking the bacterial suspensions at frequent intervals should be emphasized. Aging sometimes renders such antigens hyperagglutinable; this should be suspected if a number of unexplained positive results are found

C. Complement Fixation Tests

1 *General remarks.* The specific serologic technics of greatest importance in the diagnosis of rickettsial infection are the complement fixation and agglutination tests that employ rickettsial material as antigen. In general, the first-named has been employed more widely than the last, but each is satisfactory in experienced hands. Although these methods were applied in the rickettsioses long ago,[17, 19] it was only in the early 1940's that they began to be employed frequently for clinical diagnostic work; this followed the development of methods for the preparation of materials rich in rickettsiae. Such methods became available when Castañeda[20] showed that rodent lungs were suitable for the profuse growth of *R. mooseri* and when Cox[21] demonstrated the usefulness of the yolk sac tissue of embryonated eggs for the culture of the typhus and spotted fever groups.

Diagnostic complement fixation procedures are available for each of the common rickettsial diseases except trench fever (Table 1). Type-specific antigens can be prepared which will usually permit differentiation of even closely related infections, for example, epidemic and murine typhus. On the other hand, group-specific antigens, which are suitable for differentiating infections caused by the epidemic-murine typhus group from those attributable to the spotted fever-rickettsialpox group, are less expensive but usually fail to provide exact identification of diseases resulting from rickettsiae with common antigens such as *R. prowazeki* and *R. mooseri* or *R. rickettsi*, *R akari*, and *R. conori*.[22-24] In many geographic areas only one member of a related group of rickettsiae is prevalent; here the less expensive group-specific diagnostic antigens are adequate. In certain instances in civilian medical practice, and generally in military medicine, it is of the utmost importance to determine the exact rickettsial agent responsible for illness in a given individual; under

largely responsible for the group-specific reactions. These are dissociated from ether-treated rickettsiae during repeated washing and differential centrifugation, leaving purified suspensions of organisms that serve as type-specific antigens.[22] In the epidemic-murine typhus group the type-specific antigens are heat labile and the group-specific antigen is heat stable.[26-27]

A variety of procedures has been developed for the preparation of rickettsial antigens that are type- or group-specific or that react in an intermediate fashion In this chapter only three will be described in detail, namely, one method for obtaining washed type-specific rickettsiae and two which yield materials containing soluble antigens.

2 *Preparation of type-specific washed rickettsial antigens.* The method originally employed by Plotz[22] for obtaining type-specific antigens from *R prowazeki* and *R. mooseri*, which is given in detail in the following paragraphs, embodies most of the essential steps in the preparation of washed rickettsial antigens from infected yolk sacs. Modifications of the basic procedure currently in use at the AMSGS for preparing washed suspensions of *R rickettsi*, *R. conori*, *R akari*, and *R. burneti* are then described briefly In each instance 6- to 8-day-old chick embryos are inoculated into their yolk sacs with a sufficient concentration of seed virus to cause death of some of the embryos in 4 or 5 days and to make most of them moribund on the 6th or 7th day, at which time tissues of affected but viable embryos are harvested

Plotz used as starting material crude typhus vaccine obtained by Method A of Craigie[28], which is essentially as follows:

At the time of collection, the infected yolk sacs are allowed to drain on bronze

0 85% sodium chloride in distilled water.	4 parts
Sorensen's sodium potassium phosphate buffer, pH 7.0......	1 part
Formalin (40% formaldehyde)	to 0 5%

The suspension is filtered through bronze gauze and placed in the cold room to permit the yolk cream to rise to the surface After several days the suspension is siphoned off from below the yolk layer and centrifuged at 4,000 r p m in an angle head in the refrigerator to deposit the rickettsiae along with other particulate matter present. The floating yolk cake is carefully removed and the supernatant is discarded.

Care is taken to swab adhering traces of yolk from the tube before proceeding to resuspend the deposit The deposit is resuspended in diluting fluid similar in composition to that noted above. The diluting fluid is added in the proportion of 10 cc. per original yolk sac The deposit, after thorough dispersion, is kept in the refrigerator for several days to permit sedimentation of coarse particles to occur. The supernatant, which contains rickettsiae in suspension, is processed thus:

A separatory funnel of suitable capacity is half-filled with the suspension and a half-volume of anesthetic ether is added. The funnel is shaken vigorously and extensive emulsion formation occurs. If separation of ether at the upper surface does not begin within a few minutes, a further amount of anesthetic ether should be added and incorporated by light shaking. After standing for a short period, provided care has been taken earlier to exclude the light fraction of yolk, separation occurs into three layers: (*a*) an upper layer of excess ether, (*b*) a middle emulsion layer, and (*c*) a lower aqueous layer The middle emulsion layer (*b*) contains tissue fragments and yolk particles while the lower aqueous layer (*c*) contains rickettsiae in suspension along with a reduced amount of cell debris (and appreciable amounts of soluble antigen).

Plotz[22] applied additional washing procedures to the material obtained by Craigie's method, this yielded a highly purified suspension of rickettsiae essentially free of cellular debris and soluble antigen. His procedure was essentially as follows:

The lower aqueous layer (*c*) is centrifuged in the cold in an angle centrifuge at 4,000 r p.m for one hour. The supernatant fluid, which contains soluble antigen capable of fixing complement in the presence of both epidemic and murine convalescent serum, is discarded. The sediment is resuspended in 1/10th the original volume of buffered saline and , again extracted with ½ volume of anesthetic ether. The aqueous suspension is drawn off and again centrifuged in the cold in an angle centrifuge at 4,000 r p m for one hour The supernatant is discarded and the sediment is washed similarly four times in buffered saline After each centrifugation, the sediment is easily suspended by shaking with glass beads. The final sediment is suspended in 0 2% formol saline to a volume of 50 ml. per 300 yolk sacs.

Epidemic (Breinl strain) and murine (Wilmington strain) antigens of the Plotz type are now prepared for the AMSGS by Parke, Davis and Company; they titer in the neighborhood of 1/200 and generally are used in diagnostic tests at a 1/100 dilution.

Specific CF antigens of spotted fever and rickettsialpox used at the AMSGS are now prepared by a modification of the above procedure as follows:

Viable infected eggs are removed from the incubator on the 4th or 5th day, when 5 to 10 per cent of the embryos are dead, and placed at room temperature Cox[20] pointed out that such a reduction in temperature at this time delays death of the embryo and permits continued multiplication of rickettsiae in the yolk sac and

chorioallantoic membrane tissues On the 7th day post-inoculation, the yolk sacs together with the chorioallantoic membranes are harvested by fishing them out through the opened air sac end of the egg These tissues together with a quarter or half volume of saline solution are shaken in heavy-walled glass bottles with glass beads or ground in a vapor-proof Waring blendor, using special precautions [1] The ground material is suspended in sufficient buffered saline, pH 7 2, to make a 20 per cent concentration on the basis of original weight of yolk sacs Formaldehyde solution NF (40 per cent formaldehyde) is added to give a final concentration of 0 5 per cent of the NF solution, and the material is placed at 5° C for 7 days to permit inactivation of rickettsiae The suspension is then centrifuged at 1,000 r p m. for 10 minutes in a horizontal head The midzone is drawn off from below the yolk layer and recentrifuged for 1 hour at 4,500 r p.m in an angle head in the refrigerator to deposit the rickettsiae along with other particulate matter present The supernatant fluid and floating fat are poured off, and the tubes swabbed out carefully to remove traces of yolk. The sediment is carefully homogenized with a glass rod as diluent is added drop by drop, subsequently, buffered saline containing 0 1 per cent formaldehyde solution NF is added more rapidly until the volume is equal to that of the original 20 per cent suspension This is usually a convenient place to stop the processing and store the preparation overnight in the refrigerator The suspension is next shaken vigorously with an equal volume of cold ether (90 per cent diethyl ether and 10 per cent ethyl alcohol) If separation of the emulsion is delayed more than a few minutes, a small amount of saline may be added and incorporated by light shaking, this usually hastens separation into the 3 layers described in the Craigie procedure

The aqueous suspension is again extracted, this time with ½ volume of cold alcoholic ether, after which it is transferred to a heavy-walled suction flask attached to a suction pump and the excess ether removed The suspension is then centrifuged in the cold in an angle head at 4,500 r p m for 1 hour The supernatant fluid is discarded, the tubes wiped free of any residual fat, and the sediment is then carefully resuspended in buffered saline to 1/10th the volume of the original 20 per cent suspension This suspension is shaken with washed sterile Celite [27,30] (Hyflo supercel, a Johns-Manville product) 1 gm per 40 gms of original tissue and centrifuged in an angle head in the cold at 1,000 r p.m for 30 minutes With extreme care so as not to disturb the sediment of tissue debris that has been carried down with the Celite, the supernatant fluid is pipetted off, placed in a clean tube, and centrifuged at 4,500 r p m in a refrigerated angle head The sediment is resuspended with homogenation in buffered saline to 1/100th the volume of the original 20 per cent suspension, and formaldehyde and merthiolate are added to give a final concentration of 0.02 per cent (NF solution) and 1 10,000, respectively A smear of this suspension stained by Macchiavello's technic generally shows a moderate amount of debris and a reasonable number of rickettsiae If the debris is excessive it may be removed in part by centrifugation at 1,000 r p.m for 5 minutes Antigens prepared in this manner from rickettsialpox or spotted fever organisms have titers of 1/16 to 1/64, but give nonspecific reactions at low dilutions

Suspensions of washed *R. burneti* obtained in a manner similar to that described by Plotz for *R. prowazeki* provide CF antigens with titers of about 1/64. Since soluble antigen has not been demonstrated in tissues

infected with this rickettsia, repeated washing is not necessary. Currently supplies of Q fever CF antigen for use at the AMSGS are purchased from Lederle Division of American Cyanamid Company. The strain of *R. burneti* employed in the preparation of CF diagnostic antigen is of considerable importance[31]; the Henzerling and Nine Mile strains are usually used for this purpose. The nature of the variables in strains of *R. burneti* which contribute to the value of certain of these as diagnostic antigens has been extensively studied[32,33]; however, the answer is not yet clear.

A number of investigators have employed procedures for preparing type-specific antigens from rickettsiae of the typhus-spotted fever groups that embody the principles of the Craigie-Plotz methods. An entirely different approach permitted Cohen and his colleagues[34] to obtain specific CF antigen from epidemic typhus vaccine; this included a precipitation with sodium sulfite or with phenyl hydrazine-p-sulfonic acid. The final product was soluble after lyophilization, and in preliminary studies showed promise as a type-specific diagnostic antigen.

3. *Preparation of group-specific soluble antigens.* Group-specific soluble CF antigens prepared from yolk sacs infected with *R. prowazeki* or *R. mooseri* or with *R. rickettsi* or *R. akari* will readily differentiate serum of a case of murine typhus from that of a patient with spotted fever. Thus, such antigens fill the most common laboratory diagnostic need encountered in rickettsial diseases in the United States. When made from the agents of epidemic and murine typhus, however, they cannot be relied upon to differentiate between infection with *R. prowazeki* and *R. mooseri* because of the large amount of common antigen present in such materials processed from egg tissues inoculated with either rickettsia. On the other hand, group-specific CF antigens made from yolk sacs infected with *R. rickettsi* and *R. akari* sometimes contain so little spotted fever group antigen that they may permit serologic differentiation of sera of patients with spotted fever and rickettsialpox. The author's experience with soluble spotted fever and rickettsialpox CF antigens, both those prepared at the AMSGS and those purchased commercially, is that each lot must be assayed with standardized human convalescent serum in order to determine whether it is to be used as a group- or type-specific antigen in diagnostic tests.

The two methods outlined below yield soluble group-specific antigens for diagnosis of the epidemic-murine and spotted fever–rickettsialpox

complexes. Both methods were originally applied to yolk sacs infected with *R. tsutsugamushi.*

The procedure of Topping and Shepard[35] modified for use at the AMSGS is as follows:

Infected yolk sacs (as few as 20 may be used) are squeezed free of yolk, ground with a small amount of sterile distilled water, and placed in a thick-walled pyrex bottle at −20° C Such freezing disrupts cells and rickettsiae and provides a suitable method of storing the material for days or months without loss of antigenic activity After thawing, the tissues are extracted with 10 volumes of cold alcohol-ether The mixture is thoroughly shaken in the cold (4° C) for 30 minutes to 1 hour The ether becomes deep yellow, and after standing a few moments a reddish mass of tissue falls to the bottom of the bottle The ether is decanted as completely as possible and the tissue repeatedly washed, usually 4 or 5 times with cold ether, until yellow material no longer dissolves Approximately 1 ml of sterile distilled water is added for each gm of original yolk sac tissue, and the mixture is thoroughly shaken The residual ether is removed under partial vacuum and the tissue suspension stored overnight in the refrigerator The next morning it is centrifuged at about 3,000 r p m for 30 minutes in a refrigerated angle head machine and the supernatant further clarified by spinning at 13,000 r p m for 30 minutes The clear red supernatant is a satisfactory antigen Merthiolate solution is added to a final concentration of 1 10,000 The antigen is bottled in 10 ml volumes and stored at −20° C When maintained frozen it is stable for several years but if kept at 4° C, the titer diminishes appreciably in a few months It should be noted that in this method the material is infectious when originally treated with ether Ether inactivates the organisms, however, great care must be taken during the early steps to avoid infection of laboratory personnel

Wolfe, Vanderscheer, Clancy, and Cox[36] prepared 50 per cent suspensions of infected yolk sac in distilled water containing 0 8 per cent phenol and stored these at 4° C for 48 hours. The suspension, now noninfectious, was lyophilized and the dried material pulverized. It was then extracted at 4° C with several changes of ether over a period of hours The ether was removed by high vacuum, and the powder resuspended in sufficient saline solution containing 0.3 per cent phenol to equal the volume of the original tissue suspension. The suspension was shaken occasionally during storage at 4° C. for the next 3 to 5 days and then centrifuged in an angle machine at 3,000 r p m for 30 minutes The resultant supernatant fluid served as antigen.

Complement-fixing antigens prepared by the methods of Topping and Shepard[35] and of Wolfe *et al*[36] are sufficiently free of yolk lipids so that they do not ordinarily react with Wassermann positive sera Such is not the case with commercial epidemic typhus vaccine which can be

used in an emergency as a soluble type, complement-fixing antigen for serologic diagnosis of the epidemic-murine complex.[37] If vaccine is used as CF antigen then each serum examined must also be subjected to one of the standard tests for syphilis.

4. *Technic for complement fixation.* The CF technic used at the AMSGS for diagnosis of rickettsial diseases is as follows:

The complement titration is carried out with varying quantities, that is, 0.08 ml. to 0.26 ml with increments of 0.02 ml of a 1/30 dilution of commercial lyophilized guinea pig serum. The hemolytic system consists of equal parts of 3 per cent suspension of washed sheep erythrocytes and a solution containing 3 minimal hemolytic doses (MHD) of antisheep erythrocyte hemolysin in each 0.25 ml.

The titration is performed as follows:

Tube No.	1	2	3	4	5	6	7	8	9
Complement, 1/30	0 08 ml	0 10	0.12	0 14	0.16	0 18	0 20	0 22	0 24
Saline, 0 85 per cent	0 67 ml.	0 65	0 63	0 61	0.59	0.57	0 55	0.53	0 51
Antigen, 2 units	0 25 ml.	0 25	0 25	0 25	0.25	0 25	0 25	0 25	0.25
Sensitized R B.C	0.50 ml.	0.50	0.50	0 50	0 50	0 50	0 50	0.50	0.50

Incubate 37° C for 30 minutes

The 1st tube showing complete hemolysis is taken as the exact unit and the next higher amount as a full unit. Two full units of complement determined in the presence of antigen and contained in 0 5 ml. of 0.85 per cent saline are used in the test In the test for CF antibodies the serum is inactivated at 56° C. for 30 minutes Quarter ml. amounts of antigen, at a dilution which represents 2 units,* are mixed with 0.25 ml volumes of serial 2-fold dilutions of serum, and then 0 5 ml. of diluted complement is added The mixtures are incubated at 4° C. overnight The following morning 0.5 ml amounts of the sensitized cell suspension are added The test materials are incubated at 37° C. for 30 minutes, when final readings are made Titration end points are estimated on the basis of the last tube showing complete or 3 plus fixation, and the titer is taken as the dilution of serum originally added to that tube In each test, known positive and negative sera are titrated in the same manner as the unknown sera This titration serves as a control on the activity of the hemolytic system and permits a comparison of the data obtained from one test to another. A complement titration in the presence of antigen is included in each test to determine the amount of complement available after incubation In addition, the system is checked further by retitrating the diluted antigen actually employed in each test;

* An antigen unit is the smallest amount that gives a complete fixation with 2 full units of complement in the presence of 4 units of antibody.

for this, a 0 25 ml volume containing the presumed 2 units of antigen along with a ½, ¼, and ⅛ dilution of this are tested with 4 units of antibody

Diagnostic CF antigens are standardized immediately after preparation and at intervals of several months For this, serial dilutions of antigen, usually ¼ to 1/128, are mixed with 4 CF units of previously standardized homologous antiserum and 2 full units of complement After overnight incubation in the cold the hemolytic system is added Essential controls not ordinarily incorporated in a CF test include titration of antigen in the presence of a 1/10 dilution of normal human serum and in saline solution alone Furthermore, when type specific antigens are being assayed the test must include standardized antisera from men or animals infected with closely related rickettsiae Thus, the assay of an epidemic typhus antigen includes box titrations with epidemic and murine antisera

D Rickettsial Agglutination Tests

1. *General remarks.* Rickettsial agglutination technics are suitable for diagnostic purposes in epidemic and murine typhus[22,38,39] and in Q fever[40,41] Except under special circumstances, the author prefers CF methods. Antigens for this work usually are prepared from infected yolk sac tissue, but suspensions of typhus organisms obtained from infected mouse lungs give satisfactory results Slide and capillary technics[10,42-47] may be used for rickettsial agglutination, but a test tube method is usually preferable for diagnosis of human infection Technical difficulties that prevent the easy preparation of rich, purified suspensions of rickettsiae are undoubtedly responsible for the failure to employ this technic more extensively with members of the spotted fever group or in North Queensland tick typhus and scrub typhus, however, it has been used in Rocky Mountain spotted fever[18,30,44]

2 *Macroscopic technic.* The macroscopic rickettsial agglutination technic of Plotz and his associates[22] has been used at the AMSGS for the past 12 years It employs purified suspensions of *R. prowazeki* and *R. mooseri* prepared in the manner described above for complement-fixing antigens The agglutinating suspensions used in the test represent a 1/6 to 1/15 dilution of a stock suspension of rickettsiae. Thus, the antigen is used in a more concentrated form in the agglutination test than in the complement fixation technic, and each tube in the test receives the equivalent of about 10 complement-fixing units of antigen. The procedure[22] now in use at the AMSGS is as follows.

After various attempts to devise a method to give more reproducible end-points, it was found that the use of conical tubes promoted a greater aggregation of particles and greatly facilitated reading While smaller tubes may be used, it has been found that the 3 ml conical pyrex centrifuge tubes (Corning Glass Works, Catalog

No 8060) measuring 10 mm × 65 mm, have given optimal results. Using these tubes, the aggregation of rickettsiae was firm and not easily dispersed It was not necessary to centrifuge the tubes or to examine stained preparations in order to demonstrate agglutination. The test was extremely easy to read, especially when examined by artificial light. When the tests were read by two or more observers, comparable end-points were recorded.

One technical detail of importance must be noted. It was necessary to add normal human serum in sufficient quantity to give a concentration of 1:200 (0.5 per cent) in the antigen preparation to be used This small amount of serum was sufficient to prevent spontaneous agglutination of the rickettsiae.

Serial dilutions of serum were made in physiologic saline and distributed in 0 25 ml amounts. To each dilution of serum was added 0.25 ml of rickettsial suspension The mixture was then thoroughly shaken and placed in a water bath at 42° C for 4 hours followed by storage in the icebox at 4° C for another 16 to 18 hours when the test was read. A test was recorded as "complete" agglutination when the clumps had settled to the bottom of the tube, leaving a clear supernate, while a "partial" agglutination was one where definite clumping had occurred and settled out, but the supernate remained slightly cloudy. Only complete and partial agglutinations were recorded as positive and all titers represented final dilution. Positive and negative controls were included with each test.

3. *Slide technics.* Castañeda[43] prepared, from lungs of intranasally infected mice, suspensions of washed *R. prowazcki* and *R. mooseri* which had a turbidity corresponding to 15 mm. of the Gates loop. Lots of 20 mice usually yielded 5 to 10 ml. of antigen. Dilutions of human serum to be tested were prepared in test tubes covering the range from 1/80 to 1/640 Drops of antigen were placed on an ordinary slide with a bacterial loop of 4 mm. diameter, and the dilutions of serum added with the same loop beginning with the highest and progressing to the lowest dilution. The slides containing the mixtures were arranged in petri dishes, each containing a piece of moist cotton, and placed on a phonograph plate which rotated at 15 r p m. and was held on a 15° inclination. Readings were made in 5 to 10 minutes. The results obtained in slide agglutination tests were comparable to those obtained in complement fixation tests in which the same antigens and sera were used.

Fitzpatrick[42] prepared suspensions of *R. prowazcki* and *R. mooseri* from infected yolk sacs by a technic similar to Method A of Craigie[28] (section III, C, 2, above). Dilutions of the serum to be examined were made in test tubes, and 1 drop of each dilution was transferred with a capillary pipette to the depressions in a glass slide An equal amount of rickettsial suspension was added to each concavity, and the slides were rotated by hand and placed on moist paper in a petri dish The dishes

were held in an incubator at 40° C. for 5 hours and then were kept overnight in a refrigerator Readings were made with the aid of a hand lens. Cross-agglutination occurred with convalescent human sera and the 2 antigens, but somewhat higher titers were obtained with the homologous antigen

4. *Capillary technic.* Luoto developed a capillary agglutination test for field use in diagnosing Q fever in cattle.[46] Suspensions of washed *R. burneti* were stained for several days with hematoxylin, after which the antigen was rewashed and standardized, for details see Reference 46.

Capillary tubes 9 cm in length were used in performing the test Antigen was drawn into 1/3 of the tube, after which it was filled with the serum to be tested The ends were sealed with clay or wax, and the tubes were incubated in a vertical position with the antigen suspensions on the bottom After 2 hours at 37° C. or 4 hours at room temperature, the stained agglomerates of *R burneti* were visible macroscopically in the mixtures containing positive sera

In Luoto's hands this agglutination technic gave reproducible results that were as specific as those obtained by the usual CF procedure, however, the end point titers were somewhat higher Recently, Luoto and Mason[47] have applied this method to bovine milk and found it as reliable as the serologic tests in detecting infected cows.

E. Interpretation of Results of Complement Fixation and Agglutination Tests

In the diagnosis of rickettsial diseases, as elsewhere, it is essential to demonstrate a rise in specific antibodies during convalescence If this is done, it is unnecessary to quibble about the significance of positive results obtained with low dilutions of serum. Since serial specimens are not always received, however, it is necessary to set certain minimal levels as having significance in a single test In general, a titer of 1/10 (original dilution of serum) is significant in complement fixation tests in which washed rickettsial antigens are used At some time during convalescence in most of the rickettsial diseases, specific complement fixation titers well above 1/200 are usually obtained Rickettsial agglutination titers above 1/25 to 1/40 are probably significant in epidemic and murine typhus, whereas titers greater than 1/5 have been considered of diagnostic value in Q fever

The interpretation of the results of rickettsial complement fixation

and agglutination tests rests on more extensive knowledge in epidemic and murine typhus than in the other rickettsial diseases. The sera of individuals who have been repeatedly injected with epidemic typhus fever vaccine generally show complement fixation titers of 1/4 to 1/32 with epidemic antigen. Such individuals do not develop increased titers when they suffer from febrile illnesses of nontyphus etiology.[48] When such vaccinated persons become infected with either the homologous or heterologous typhus organism, however, they show a prompt rise in epidemic and murine complement-fixing antibodies.[14,49,50] The amount of crossing in such tests may be sufficient to preclude a differential diagnosis between epidemic and murine typhus. Nevertheless, a serologic diagnosis can generally be made in these individuals by means of the rickettsial agglutination test, since higher titers are obtained with antigens prepared from the infecting organism.

A word of caution should be injected regarding the interpretation of

fection with a member of the immediate family of agents. However, the laboratory worker should be conservative in his attempts to differentiate the particular agent responsible for the patient's illness. Results of such tests should be reported as "Epidemic-Murine Group, CF titer . . . " or "Spotted Fever Group, CF titer . . . "

Most of the observations on time of appearance of rickettsial antibodies were accumulated before the era of specific antibiotic therapy. There is some evidence that under certain conditions antibody production may be delayed in patients who receive specific therapy early in the course of disease.[51] Until adequate experience is obtained with the rickettsioses under present conditions of treatment, it is probably desirable to examine a late convalescent serum obtained 4 to 6 weeks after onset of illness, if the laboratory diagnosis has not been established by that time.

F. Antiglobulin Sensitization Test

Coombs and Stoker[52] in their search for more sensitive technics for the detection of Q fever antibodies employed the principles of the antiglobulin sensitization test which had been introduced for the detection of incomplete Rh antibodies. These authors employed washed suspensions of *R. burneti* in their work which embraced complement fixation and direct agglutination technics as well as the indirect procedure. The

description given below of the technics for the last 2 tests is taken directly from their article [52]

Direct agglutination tests—Serial dilutions of the antisera [convalescent human serum] were made in 0.1 ml. volumes of saline in small round-bottomed tubes of internal dimensions 7 × 50 mm An equal volume of antigen diluted 1 in 5 was added to each tube and to a control tube containing saline only. The tests were incubated overnight in a 37° C water-bath, in which the water level was so arranged as to cause convection currents in the tubes The readings were made, in indirect light against a dark background, with the aid of a hand lens.

37° C the tubes were centrifuged in an angle centrifuge at 4000 revolutions per

washing procedure was then repeated

adsorbed antibody globulin The tubes were then re-incubated in a water-bath at 37° C. Agglutination of the sensitised rickettsiae could be observed after one–two hours, but the agglutination was at its maximum after five hours Readings could be made conveniently after incubation overnight The titre of Q-fever antibodies in an antiserum was expressed as the highest dilution of the serum which so sensitised the rickettsiae that definite agglutination occurred with the anti-globulin serum

In these early experimental investigations each test was controlled by setting up a parallel series of tubes and adding normal rabbit serum instead of rabbit anti-globulin serum to the washed sensitised rickettsial suspensions The agglutination titre observed in these controls corresponded in each case to that found by direct agglutination

During the washing procedure great care should be taken to avoid contamination of the clean saline with human serum, since traces of globulin in the saline of the final suspension might easily neutralise the agglutinating action of the anti-globulin serum

The authors found the antiglobulin sensitization test to be specific and more sensitive than the complement fixation or agglutination technics, that is, it gave higher titers with human convalescent sera. However, they recognized its limited value as a clinical diagnostic tool because of the time-consuming nature of the procedure and the relatively large amounts of antigen required. On the other hand, the high degree of sensitivity of the technic made it of potential practical importance for detecting small amounts of Q antibodies in bulk pools of cows' milk [53]

G. Other Serologic Procedures of Laboratory Importance

There are several types of serologic tests which are of assistance to the laboratory worker in carrying out various fundamental studies but which are of little importance in the diagnosis of human disease. Among these are the antitoxin titration, the hemagglutination and hemolysin tests, the neutralization test, and the precipitin reaction. Needless to say, the complement fixation and agglutination technics are as valuable in investigative work as they are in clinical diagnosis.

1. *Antitoxin titration.* A toxic substance, capable of causing death of mice within a few hours, is found in yolk sacs which are rich in *R. mooseri*,[54] *R. prowazeki*,[25,55] *R. tsutsugamushi*,[56] *R. rickettsi*,[57] and *R. conori*.[57] The toxins of epidemic and murine typhus are closely related but not identical[58]; the same is true for those of Rocky Mountain spotted fever and African tick fever.[57] Toxins of the typhus group, however, are unrelated to those of the spotted fever group or to that of scrub typhus. The sera of men and animals recovered from infection with these agents contain immune substances which are capable of neutralizing the toxic effect.[25 54-57] While the demonstration of antitoxin in the serum of convalescents might be used as a diagnostic procedure, this type of test has not had wide application because of the technical difficulties associated with it Nevertheless, the antitoxin test played a considerable role in the development of typhus vaccine[25,26] and is still used in the standard assay of epidemic typhus vaccine.[59]

2. *Hemagglutination and hemolysis.* Several kinds of soluble, serologically active substances have been used to coat erythrocytes and thus render them agglutinable by rickettsial antibodies. This basic principle, which has found wide application in microbiology since its employment by Keogh and his colleagues,[60] was introduced into rickettsial problems by O'Connor and MacDonald.[61] The last-named workers detected specific antigen in the urine of patients with scrub typhus. They employed a carefully balanced system consisting of convalescent human serum and chicken erythrocytes coated with polysaccharide extracted from proteus OX-K. Urine to be tested was boiled and then mixed with serial dilutions of the standard serum, after which the sensitized cells were added. Antigen present in the urine combined with the antibody, which was then unavailable for agglutination of sensitized cells; an estimate of the quantity of antigen in urine was possible by this method. In the hands of O'Connor and MacDonald the procedure showed promise as a means

for early diagnosis of scrub typhus However, the author has so far been unable to obtain sufficiently consistent results under field conditions with the method to consider it applicable on a routine basis in this disease.

Chang, Murray, and Snyder[62 64] performed hemagglutination tests using appropriate convalescent sera and human O erythrocytes sensitized with extracts of *R prowazeki*, *R mooseri*, *R. rickettsi*, *R. conori*, or *R. akari*; the extracts were obtained by heating the organisms in an alkaline solution. The results correlated well with those displayed by the same sera in CF and Weil-Felix tests, and in rickettsial agglutination tests when these were performed The erythrocyte sensitizing substances (ESS) of these authors did not react in direct CF or precipitin tests, and antibodies against the ESS of *R prowazeki* appeared to be different from those which agglutinate proteus *OX-19* or epidemic typhus organisms or which fix complement with type-specific epidemic typhus antigens ESS has a group-specific serologic activity, however, similar to that of soluble CF antigens of the typhus and spotted fever families of agents. Moreover, Downs and her associates[65] found that addition of group-specific (soluble) antigen, prepared from *R mooseri*, to antisera inhibited their capacity to agglutinate erythrocytes treated with ESS. This points to a close relationship between ESS and soluble antigen and adds weight to the idea that ESS may be a degradation product of soluble antigen. Chang and his colleagues[64] consider the ESS test more economical and technically simpler than the CF procedure for the diagnosis of the typhus and spotted fevers in man and regard it as equal to the CF method in reliability It may be reiterated that the ESS results are group specific, not type specific.

Recently Downs and her associates[65] extended the work on ESS by demonstrating a related heat stable antigen in tissues of mice infected with murine typhus. This antigen, which occurs as early as 3 days after infection, reacts with murine antisera specifically inhibiting its capacity to agglutinate red cells sensitized with Chang's ESS. The tissue substance of Downs resembles ESS in a number of its properties, but unlike ESS it fails to sensitize erythrocytes Extension of the approaches of O'Connor and of Downs are warranted in the hope of obtaining a useful method for the early diagnosis of the rickettsioses.

Highly infectious preparations of *R. prowazeki* and *R. mooseri* induce in vitro hemolysis of erythrocytes of rabbits and sheep but do not affect red cells of mice, cotton rats, and guinea pigs.[66] The specific inhibition of such hemolysis by antisera forms the basis for another type of sero-

logic procedure.[66] However, the hemolytic phenomenon itself, rather than its use in serology, has been the main object of interest. Snyder and his associates[67,68] found that the hemolytic activity of a preparation of rickettsiae—see reference 67 for technic of rapid hemolytic test—could be correlated with its in vitro metabolic activity and its content of mouse lethal toxin but that all 3 of these properties could be disassociated from infectivity. Paterson *et al.*[69] demonstrated that in vivo hemolysis followed injection of *R. mooseri* into rabbits but did not occur in rats. Moreover, such lysis contributed to the fatal toxemia in the former host.

3. *Neutralization tests.* Neutralization tests or protection tests have been used with most of the rickettsial agents; the principle here is the same as in neutralization tests employed with viral agents. Thus, antibody is added to a suspension of organisms, and the mixture is injected into susceptible animals. If the animals fail to show evidence of infection, the organism is said to be neutralized by the antibody. In general, the results obtained in this technic are not as clean-cut as with some of the viral agents, and while the test is useful under certain conditions, it is not employed too frequently because other simpler methods are available which usually may be substituted for it.

4. *Precipitin reaction.* The precipitation reaction has been employed in certain of the studies with soluble antigens of rickettsiae[25] where complement fixation was not readily applicable. One of the limitations of the precipitation method in rickettsial work is the difficulty in obtaining relatively pure soluble antigens; it will be recalled that rickettsiae only grow in the presence of living tissue, and the occurrence of large amounts of extraneous protein in preparations of such infected tissue make it difficult to obtain pure preparations of soluble antigen.

Investigators in Mexico and Russia have demonstrated specific serologically active substances in the urine and blood of patients in the acute stage of typhus fever.[70,71,72] The precipitin test, the coated colloid particle agglutination reaction, and the complement fixation technic have been used to detect the antigen, or antigens, which appear in the body fluids. These studies show promise of providing a diagnostic method applicable early in the disease, but additional work along these lines is indicated.

IV REFERENCES

1 Smadel, J. E The Hazard of Acquiring Virus and Rickettsial Diseases in the Laboratory *Am J Pub Health*, 41 788–795, 1951

2 Parker, R R, and Steinhaus, E A American and Australian Q Fevers Persistence of the Infectious Agents in Guinea Pig Tissues after Defervescence *Pub Health Rep*, 58 523–527, 1943

3 Burnet, F M, and Freeman, M Note on a Series of Laboratory Infections with the Rickettsia of "Q" Fever *M J Australia*, 1 11–12, 1939

4 Derrick, E H "Q" Fever, New Fever Entity, Clinical Features, Diagnosis and Laboratory Investigation *M J Australia*, 2 281–299, 1937

5 Rose, H M The Clinical Manifestations and Laboratory Diagnosis of Rickettsialpox *Ann Int Med*, 31 871–883, 1949

6 Snyder, J C, and Wheeler, C M The Experimental Infection of the Human Body Louse, *Pediculus humanus corporis*, with Murine and Epidemic Louse-Borne Typhus Strains *J Exper Med*, 82 1–20, 1945

7 Jellison, W L, and Philip, C B Technique for Routine and Experimental Feeding of Certain Ixodid Ticks on Guinea Pigs and Rabbits *Pub Health Rep*, 48 1081–1082, 1933

8 Snyder, J C The Typhus Fevers Chapter 35 in the *Viral and Rickettsial Infections of Man*, 2d ed Edited by Rivers, T M Philadelphia Lippincott, 1952, p 591

9 Felix, A Serological Types of Typhus Virus and Corresponding Types of Proteus *Tr Roy Soc Trop Med and Hyg*, 27 147–172, 1933

10 Castañeda, M R. Clinical and Experimental Aspects of Exanthematous Typhus in the Tropical Regions of America. Aids to Diagnosis *Puerto Rico J Pub Health and Trop Med*, 18 165–174, 1942

11 Welch, H, and Bengtson, I A Typhus Fevers and Other Rickettsial Infections In *Diagnostic Procedures and Reagents*, 2d ed New York, N Y Am Pub Health A 1945, pp 232–246

12 Plotz, H The Rickettsiae Chapter 32 in *Laboratory Methods of the United States*

13

14

J Immunol, 53 15–30, 1946

15 Murray, E S, Baehr, G, Schwartzman, G, Mandelbaum, R A, Rosenthal, N, Doane, J C, Weiss, L B, Cohen, S, and Snyder, J C Brill's Disease I Clinical and Laboratory Diagnosis *J A M A*, 142 1059–1066, 1950

16 Murray, E. S, and Snyder, J C Brill-Zinsser Disease The Interepidemic Reservoir of Epidemic Louse-Borne Typhus Fever. (ATTI DEL) Proc VI *Internat Cong Microb* Rome Vol 4, Sez XI, Pag 31–44, 1953

17 Otto, R, and Dietrich, A Beitrage zur "Rickettsien"-Frage *Deutsche med. Wchnschr*, 43 577–580, 1917

18 Ricketts, H T A Micro-Organism Which Apparently Has a Specific Relationship to Rocky Mountain Spotted Fever *J A M A*, 52 379–380, 1909.

19 Zinsser, H, and Castañeda, M R. Studies on Typhus Fever. IX On the Serum Reactions of Mexican and European Typhus Rickettsia *J Exper Med.*, 56 455–467, 1932

20 Castañeda, M R Experimental Pneumonia Produced by Typhus Rickettsiae *Am J Path*, 15 467–476, 1939

21 Cox, H R Use of Yolk Sac of Developing Chick Embryo as Medium for Growing Rickettsiae of Rocky Mountain Spotted Fever and Typhus Groups. *Pub Health Rep*, 53 2241–2247, 1938

22. Plotz, H., Bennett, B. L., Wertman, K., Snyder, M. J., and Gauld, R. L. The Serological Pattern in Typhus Fever. I. Epidemic. *Am. J. Hyg.*, 47:150–165, 1948.

23.

24.
1605–1614, 1946.

25. Topping, N. H., Bengtson, I. A., Henderson, R. G., Shepard, C. C., and Shear, M. J. Studies of Typhus Fever. *Nat. Inst. Health Bull.*, No. 183, 1945.

26. Craigie, J., Watson, D. W., Clark, E. M., and Malcolmson, M. E. The Serological Relationship of Rickettsiae of Epidemic and Murine Typhus. *Canad. J. Research*, Sect. E, 24:84–103, 1946.

27. Fulton, F., and Begg, A. M. Chemotherapeutic and Other Studies of Typhus. V. The Antigenic Structure of Typhus Rickettsiae. *Medical Research Council, Special Report Series*, No. 255, London, 1946, pp. 163–191.

28. Craigie, J. Application and Control of Ethyl-Ether-Water Interface Effects to the Separation of Rickettsiae from Yolk Sac Suspensions. *Canad. J. Research*, Sect. E., 23:104–114, 1945.

29. Cox, H. R. Growth of Viruses and Rickettsiae in the Developing Chick Embryo. *Ann. New York Acad. Sc.*, 55:236–247, 1952.

30. Shepard, C. C., and Topping, N. H. Preparation of Suspensions of Rickettsiae from Infected Yolk Sacs Without the Use of Ether. *J. Immunol.*, 55:97–102, 1947.

31.

32.

33. Stoker, M. G. P. Variation in Complement-Fixing Activity of *Rickettsia burneti* during Egg Adaptation. *J. Hyg.*, 51:311–321, 1953.

34. Cohen, S. S., Chambers, L. A., and Clawson, J. R. Studies on Commercial Typhus Vaccines. III. The Concentration and Isolation of the Rickettsia-Specific Soluble Antigen of Commercial Typhus Vaccine. *J. Immunol.*, 65:465–473, 1950.

35. Topping, N. H., and Shepard, C. C. The Preparation of Antigens from Yolk Sacs Infected with Rickettsiae. *Pub. Health Rep.*, 61:701–707, 1946.

36. Wolfe, D. M., Vanderscheer, J., Clancy, C. F., and Cox, H. R. A Method for the Preparation of Complement-Fixing Antigens in a Study of Experimental Tsutsugamushi Disease (Scrub Typhus). *J. Bact.*, 51:247–255, 1946.

37. Reynolds, F. H. K., and Pollard, M. The Employment of a Rickettsial Vaccine for Antigen in the Diagnostic Complement-Fixation Test. *Am. J. Trop. Med.*, 23:321–323, 1943.

38. Stuart-Harris, C. H., Rettie, G. K. C., and Oliver, J. O. Rickettsial Agglutination Studies in Typhus Fever. *Lancet*, 2:537–538, 1943.

39. van Rooyen, C. E., and Bearcroft, W. G. C. Typhus Rickettsial Agglutination Tests in the Middle East Forces and Egypt. *Edinburgh M. J.*, 50:257–272, 1943.

40. The Commission on Acute Respiratory Diseases, Fort Bragg, North Carolina. Identification and Characteristics of the Balkan Grippe Strain of *Rickettsia burneti*. *Am. J. Hyg.*, 44:110–122, 1946.

41. Lennette, E. H., Clark, W. H., Jensen, F. W., and Toomb, C. J. Q Fever Studies. XV. Development and Persistence in Man of Complement-Fixing and Agglutinating Antibodies to *Coxiella burnetii*. *J. Immunol.*, 68:591–598, 1952.

42. Fitzpatrick, F. K. Studies on Rickettsial Agglutination in Typhus. *J. Lab. and Clin. Med.*, 30:577–586, 1945.

43. Giroud, P., and Giroud, M. L. Agglutination des Rickettsies, Test de Séro-Protection et Réaction d'Hypersensibilité Cutanée. *Bull. Soc. Path. Exot.*, 37:84–93, 1944.

44. Fitzpatrick, F., and Hampil, B. Immunological Reactions in Rickettsial Diseases with Special Reference to the Time of Appearance of Antibodies *Am J Pub Health*, 31 1301–1305, 1941.

45 Castañeda, M R Differentiation of Typhus Strains by Slide-Agglutinative Tests *J. Immunol*, 50 179–183, 1945.

46 Luoto, L. A Capillary Agglutination Test for Bovine Q Fever. *J Immunol*, 71 226-231, 1953.

47 Luoto, L, and Mason, D M. An Agglutination Test for Bovine Q Fever Performed on Milk Samples *J Immunol*, *74 222–227, 1955*

48 Zarafonetis, C J D Serologic Studies in Typhus-Vaccinated Individuals II The Effect of Non-Typhus Fevers on the Weil-Felix and Complement-Fixing Antibodies *J Immunol*, 51 375-388, 1945.

49. Smadel, J E Complement Fixation and Agglutination Reactions in Rickettsial Diseases In *The Rickettsial Diseases of Man* Washington, Am Assn Adv Sc, 1948, pp 190–197

50 Plotz, H, and Wertman, K Modification of Serological Response to Infection with Murine Typhus by Previous Immunization with Epidemic Typhus Vaccine *Proc Soc Exper Biol and Med*, 59 248–251, 1945

51 Smadel, J. E Influence of Antibiotics on Immunologic Responses in Scrub Typhus *Am J Med*, 17 246–258, 1954

52 Coombs, R R A, and Stoker, M G P Detection of Q-Fever Antibodies by the Anti-globulin Sensitisation Test. *Lancet* 2 15–17, 1951

53 Stoker, M G P, and Marmion, B P Detection of *Q Fever* Antibodies in Whey by the Anti-globulin Sensitization Test and Other Techniques *J Hyg*, 50 1–11, 1952.

54 Gildemeister, E, and Haagen, E Fleckfieberstudien, Nachweis eines Toxins in Rickettsien-Eikulturen (*Rickettsia mooseri*) *Deutsche med Wchnschr*, 66 878–880, 1940

55 Otto, R, and Bickhardt, R Ueber das Gift der Fleckfieberrickettsien *Ztschr Hyg*, [illegible]

56 [illegible]

57 [illegible]

58 Hamilton, H L Specificity of the Toxic Factors Associated with the Epidemic and [illegible]

59 [illegible]

60 [illegible]

61 [illegible]

62 [illegible]

63 [illegible]

64 [illegible] *73 8–15, 1954*

65 Downs, C M, Fevurly, J, and Meyer, M. M Studies on Hemagglutination Inhibition Phenomena I The Presence of Inhibiting Antigen in Typhus-Infected Animals *J Immunol*, 75 35–42, 1955.

66 Clarke, D H, and Fox, J P The Phenomenon of in Vitro Hemolysis Produced by the Rickettsiae of Typhus Fever, with a Note on the Mechanism of Rickettsial Toxicity in Mice *J. Exper Med*, 88 25–41, 1948

67. [illegible]

68 [illegible]
723, 1954.

69 Paterson, P Y, Wisseman, C. L, Jr., and Smadel, J E. Studies of Rickettsial Toxins. I. Role of Hemolysis in Fatal Toxemia of Rabbits and Rats. *J. Immunol.*, 72 12–23, 1954.

70 Leon, A. P Precipitacion de Sueros Anti-Tifo por la Orina de Enfermos de Tifo Exantemático, Una Nueva Reacción Serológica para el Diagnóstico del Tifo. *Rev Inst. salub y enferm. trop*, 3 201–208, 1942.

71 Leon, A. P., and Cano, C. La Reacción de "Fijación del Complemento Inversa" en el Diagnostico del Tifo Exantemático *Rev Inst. salub y. enferm. trop.*, 6:167–172, 1945.

72 Smorodintzeff, A A, and Fradkina, R. V. Slide Agglutination Test for Rapid Diagnosis of Pre-eruptive Typhus Fever. *Proc Soc Exper. Biol. and Med.*, 56 93–94, 1944.

FIG 1 Bench arrangement for inoculation of embryonated eggs by the yolk sac route The towel is damp with diluted lysol solution The bowl with antiseptic is ready to receive contaminated instruments, etc , these will be decontaminated by boiling immediately after the procedure is completed



FIG 2 Bench arrangement for harvesting and triturating of yolk sac tissue See text d description

PLAN FOR VIRAL ISOLATION STUDIES ON CLINICAL MATERIALS

(Department of Virus and Rickettsial Diseases, AMSGS, 1955)

TABLE I

SPECIMENS AND AGENTS CONSIDERED IN DIAGNOSTIC VIRAL STUDIES

Type Specimen	Viruses Given Primary Consideration
Central nervous system tissue	Poliomyelitis *Arthropod-borne encephalitis* Lymphocytic choriomeningitis Rabies *Herpes simplex* Coxsackie and "Orphan"—Echo groups (?)
Cerebrospinal fluid or blood	Usually unsatisfactory except in lymphocytic choriomeningitis, Colorado tick fever, yellow fever and dengue
Throat washings, sputum, saliva, oropharyngeal *and conjunctival swabs*	Influenza "RI-APC-ARD" (Adeno group) Psittacosis* Herpes simplex Mumps Newcastle disease Poliomyelitis† Coxsackie† "Orphans"† Echo
Stool	*Poliomyelitis* Coxsackie "Orphans"—Echo
Vesicular fluid and crusts	Variola Vaccinia *Herpes simplex*
Buboes and lymph nodes	Lymphogranuloma venereum*

* Usual antibiotics omitted from specimens suspected of containing Psitt—LV group.
† Stool is preferred specimen for isolation of these viruses from nonfatal cases

TABLE II

VIRAL DISEASES IN WHICH DIAGNOSTIC ISOLATION METHODS ARE UNAVAILABLE OR NOT GENERALLY EMPLOYED

U—Infectious mononucleosis
N—Measles
N—Primary atypical pneumonia (cold and Strep MG agglutinins)
U—Rubella
N or U—Trachoma
N—Varicella

U—Unavailable
N—Not generally employed.

PLAN FOR VIRAL ISOLATION STUDIES ON CLINICAL MATERIALS *(Continued)*

TABLE III

ISOLATION AND IDENTIFICATION OF VIRUSES FROM CLINICAL MATERIAL (AMERICAN)

Laboratory Host	Suckling Mice (SM)	Mice	Guinea Pigs (GP)
Hosts used primarily to detect	Coxsackie group Herpes simplex Colorado tick fever (CTF) Dengue Also numerous viruses pathogenic for adult mice	Arthropod-borne neurotropic viruses Lymphocytic choriomeningitis (LCM) Rabies Psittacosis-lymphogranuloma venereum* Encephalomyocarditis (EMC) Yellow fever	Lymphocytic choriomeningitis (LCM)
Inoculation	Combined IC and IP inoculation 2 SM families 24 hours old	IC inoculation 6–10 mice, 3–4 weeks old (10–12 gm)	Combined IC and IP inoculation 2 young GP (approx. 300 gm)
Observation	21-day observation for death or obvious disease	Same as under SM	Daily rectal temp for 21 days (hold for 6 weeks if tuberculosis is considered)
Passage	*Several ill animals* — pool brains and muscles and pass as above to establish transmissibility *Blind passages*—if no disease by 5-7 days, sacrifice 2 SM and pass as above At least 1 or more blind passages indicated	*Several ill animals*—pool brains and pass as above to establish transmissibility *Blind passages*—not generally indicated	*Blood, brain, or spleen*—pass from GP with fever (104° F) for 48 hrs—hold survivors for antibody tests (Fever in first 24 hrs. usually is non-specific) *Blind passages*—not generally indicated
Preliminary identification	*Host range** *Histology*— *Coxsackie* *Group A*—muscle lesions only *Group B*—involvement of CNS, fat pad, etc., variable muscle lesions *Herpes simplex* — intranuclear inclusions	*Host range** *Histology*— *Rabies*—Negri bodies	*Host range** *Serology*—CF using (1) spleen of original sick animal as antigen with LCM serum, or (2) survivors' serum with LCM antigen
Final identification	*Serology*—CF or neutralization test using SM brain and known positive sera	*Serology*—CF or neutralization test, using mouse brain as unknown against known positive sera (SM brain is frequently a better source of viral antigen) *Cross protection*—may be used for further identification	Neutralization or cross protection tests may be indicated in selected instances
Relation of agent to patient's disease	Established by demonstration of (1) appearance in patient's serum during convalescence of *antibodies against the isolate or known viral materials* or (2) an increased amount of such antibody in convalescent serum as compared with acute phase serum		

* See related chapters

Laboratory Host	Chick Embryos		Tissue Cultures (Monkey Kidney or HeLa Cell)
	Chorioallantoic membranes (CAM)	Amniotic sacs (AMN SAC)	
Hosts used primarily to detect	Herpes simplex Variola–vaccinia group	Influenza Mumps	Poliomyelitis Coxsackie group "Orphan" group—Echo RI APC ARD (adeno group) (HeLa cell preferred)
Inoculation	CAM inoculation 5–10 eggs approx 11–12 days old	Amn sac inoculation 10 eggs approx 7 days old for mumps and 10 days for flu	Inoculate 3–6 culture tubes containing sheets of cells
Observation	Harvest membranes at 48–72 hours and examine for pocks	Test individual amn fluids for HA*	Daily at 35 × magnification for cytopathogenic changes Hold cultures 14–21 days if no change
Passage	*If lesions present*—pool involved CAM and pass to establish transmissibility Use remainder of pool for identification *Blind passages*—1 blind passage	*If hemagglutination occurs*—pool positive amn fluids and pass to additional amn sacs (undiluted and 10–3) to establish transmissibility Use remainder of pool for identification *Blind passages*—if no hemagglutination, make 2–3 blind passages in amn sacs	*With cytopathogenic changes*—pass to establish transmissibility *Blind passages* — if no cytopathogenic changes, pass cells and fluid of 1–2 culture tubes at 4–10 days (1 blind passage)
Preliminary identification	*Serology*—CF using CAM as antigen and positive variola–vaccinia group serum *Histology*—herpes intranuclear inclusions	*Serology*—HAI or CF test using amn fluid with positive sera	*Serology* — tissue culture neut with 3 types polio antisera or CF with RI–APC ARD (adeno group) sera *Host range*—inoculate SM for Coxsackie group, members of RI–APC–ARD (adeno group) produce distinctive cytopathogenic changes in HeLa cultures
Final identification	*Serology*—neutralization or CF test with herpes antiserum *Host range*—differentiation between members of variola–vaccinia group	*Serology*—detailed studies for antigenic analysis of influenza strains	*Serology*—typing of RI–APC–ARD adeno by tissue culture neut Typing of Coxsackie tissue culture or SM neut or by CF *Special study*—for "orphans"—Echo
Relation of agent to patient's disease	Established by demonstration of (1) appearance in patient's serum during convalescence of antibodies against the isolate or known viral materials or (2) an increased amount of such antibody in convalescent serum as compared with acute phase serum		

* See related chapters

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